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There has been increasing evidence that high intake of dietary fats and obesity, in general, correlate with increased risk of breast cancer. The breast, as an apocrine gland shrouded in fat pads, may possess special regulatory mechanisms to protect the epithelial cells from stimulation by products generated in the rich mileu of the surroundings. Our hypothesis addresses that issue suggesting that loss of protective regulatory mechanisms in breast tissue leaves breast epithelial cells vulnerable to unregulated stimulation. Our studies have addressed regulatory mechanisms in production of bioactive lipids in breast cancer cells. We have shown, using estrogen receptor + / - breast cancer cell lines, expression of various lipoxygenase (LO) and cyclooxygenase mRNA espression. Insulin-like growth factor-I and transferrin each stimulated production of 5-LO products in 4 different breast cancer cell lines. Furthermore, inhibition of the LO pathway induced apoptosis in breast cancer cells. We identified that blocking other lipid metabolism enzymes caused cells to accumulate in Go/G1 phase of the cell cycle and subsequently induced apoptosis. A full array of these drugs were evaluated for toxicity to bone marrow cells and many were found to exhibit minor toxicity. We synthesized new classes of bioactive lipid targeting inhibitors. These water soluble, stable drugs effectively blocked proliferation in breast cancer cultures yet showed little toxicity in bone marrow cultures. Mice were given regimens for 3-10 weeks of the HPA-Na drug; necropsy revealed no toxicity other than hair loss at the site of drug administration. We are evaluating the option of pursuing IND approval. We have identified cross talk between MAP kinase pathway and arachidonic acid metabolism stimulated by IGF-I in breast cancer cells. These important mechanistic studies open up new avenues for selection of possible drug treatments and regimens to combat breast cancer.

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AIBS 2691

ARACHIDONATE METABOLISM IN BREAST CANCER CULTURES: IDENTIFICATION OF ANTOAGNISTS/ AGONIST FOR POSSIBLE INTERVENTION STRATEGIES.

PRINCIPAL INVESTIGATOR: Marti Jett, Ph.D.

(5) INTRODUCTION:

Evidence has accumulated, and was presented at the "Era of Hope" breast cancer meeting which showed that increased dietary fats correlate with increased risk of breast cancer. Additionally, an association was described connecting obesity with increased risk of breast cancer. The breast, as an apocrine gland shrouded in fat pads, may possess special regulatory mechanisms to protect the epithelial cells from stimulation by products generated in the rich mileu of the surroundings. Our hypothesis addresses that issue suggesting that loss of protective regulatory mechanisms in breast tissue leaves breast epithelial cells vulnerable to unregulated stimulation. Lipid metabolism in the normal or cancerous breast is not well understood. This research proposal was initially prompted by the publication of epidemological data suggesting that people who took low dose aspirin daily for heart disease had a decreased incidence of colon cancer relative to a control group {26}. It was not clear if these individuals had also changed their food habits and life style or if other factors might also have contributed to the observed results. In our current studies (see Appendix, manuscripts 1, & 2), use of aspirin and other cyclooxygenase inhibitors to alter lipid metabolism in breast cancer cells, stimulated rather than inhibited proliferation in numerous breast cancer cell lines. Those experiments, however, do not address the issue of whether or not aspirin could serve to prevent the onset of malignant disease (as might be inferred from the studies in which low dose aspirin intake was correlated with diminished colon cancer). Furthermore, as we had seen previously with small cell lung cancer cells, lipoxygenase, rather than cyclooxygenase inhibitors, blocked proliferation and induced apoptosis in breast cancer cells (Appendix, manuscript 1). These results can indicate various possibilities; for example, a) that certain blocked metabolites (or combinations of metabolites) are critical for proliferation, b) that diverted metabolism has caused the production of toxic metabolites, etc. Therefore, in our studies we have evaluated the levels of approximately 30 arachidonic acid metabolites alone, in the presence of a growth factor +/- inhibitors which lead to apoptosis. Many of the arachidonate metabolites have been characterized as initiating cascades of other biologically active molecules such as cytokines, activation of kinases and calcium mobilization. These activators of cellular signals have even been shown to alter nuclear receptors that directly interact with DNA to have far-reaching effects on cellular functions {44}.

Arachidonic Acid Metabolism.

Arachidonic acid is released from the Sn-2 position of phospholipids by the family of enzymes phospholipase A2 (PLA2). There are small soluble secreted PLA2's, and PLA2's which are located within the nucleus. The newly released arachidonic acid is, then, the substrate for numerous enzymes including lipoxygenases (LO); a 5-lipoxygenase activating protein (FLAP) is thought to form a complex with 5-LO and anchor it to the inner aspect of the nuclear membrane producing numerous bioactive lipids which initiate the cascades of mediator release. A simplified chart of arachidonic acid metabolism is shown in Fig. 1. The two major classes of enzymes responsible for arachidonic acid metabolism are the cyclooxygenases (COX) and lipoxygenases (LO). Cyclooxygenases produce the prostanoids and thromboxanes. The best known of these is probably prostaglandin E2, the compound involved in pain and inflammation. Other prostanoids have been implicated in cell proliferation. A multidrug-resistant breast cancer cell line{4,5}, MCF-7 ADR^{10}, has greatly increased levels of prostacyclin H synthetase {28}. Lipoxygenases alter arachidonic acid by first adding a hydroperoxy group (through a free-radical mechanism) at the so named (5-, 12- 15-, etc.) carbon position, and the resulting compounds are named hydroperoxyeicosatetraenoic acids (HPETE). The second generation compounds are hydroxyeicosatetraenoic acids (HETE's). The lipoxygenases are named for the carbon position that they modify; the 5, 12, and 15 being the most common. The 12- and 15-HETE's have been shown to stimulate cell proliferation in low concentrations {1-3}, however, they become quite toxic at higher concentrations. The 5-lipoxygenase (LO) pathway has been studied extensively because of its involvement in acute allergy symptoms, shock, adult respiratory distress syndrome{2}. We have identified a 5-LO metabolite, 5-HETE, as a possible growth (co)factor in small cell lung carcinoma and now, as will be described, have implicated it in breast cancer. This compound is also converted to lipoxins, metabolites characterized to stimulate protein kinase C. Lipoxins are abundantly produced in response to growth factors in the tumor cell systems, which we are studying. In addition, specifically hydroxylated linoleic acid has been shown to occur upon stimulation of 3T3 cells with EGF {8}. It is unclear if the same lipoxygenase utilize both arachidonic and linoleic acids.

We have used a number of inhibitors of arachidonate metabolism to manipulate the outcome of agonist/antagonist effects. We have attached two manuscripts (in final stages of preparation for submission for publication (appendix, manuscripts 1 and 2) which provide details of what we have learned about alteration of lipid metabolism in breast cancer cultures. Several of the 5-lipoxygenase inhibitors are in clinical trials for treatment of acute asthma, adult respiratory distress syndrome and arthritis {1-3, 11}. We have used some of these in studies involving toxins, etc. and have demonstrated that these regulators of bioactive lipid generation block proliferation in a battery of cultured breast cancer cells including estrogen receptor positive and negative cultures and various malignant stages of cultures. Over the course of this study, we have begun to learn that generation of these bioactive lipids is unusually high in breast cancer cultures and blocking a single enzyme involved in these cascades of reactions, while a reasonably effective anti-proliferation strategy, can be improved. Therefore, we have directed many of our studies to examine the use of heteropolyanions that prevent many of the initial electron-transfers and free-radical intermediates essential for utilization of arachidonic acid.

(6) BODY

MATERIALS:

Materials: Phenol red-free IMEM (improved minimal essential medium; BioFluids, Walkersville, MD); Complete culture fluid containing 7% fetal bovine serum, 1% MEM vitamins, appropriate growth factors (if required) and 1% antibiotics; all from BioFluids, Walkersville, MD; bioactive lipid standards and many specific inhibitors were purchased from BioMol Inc., Plymouth Meeting, PA or were gifts from Merck-Frosst, Pointe Clair, Montreal, Canada.

Cell cultures: Various breast cancer cultures including ER-positive ZR-75, T47D, and MCF-7 WT; ER-negative cultures include SKBR3, MB231, MCF-7 ADR^{10} and T47D-co cells. All were grown in phenol red-free IMEM to avoid problems with estrogen-like activities of phenol red. Cells were subcultured twice per week to prevent irreversible clumping. Cultures were discontinued after 15 subcultures and new cultures brought up from frozen stock. Cultures were tested weekly for estrogen receptor status. Normal breast epithelial cells were obtained from Dr. Vimla Band, New England Medical Center, and cultured according to instructions provided by Dr. Band.

METHODS:

1. **Establish quiescence in cultures of MCF-7 cells.** In the previous report, we showed that quiescence by serum depletion for 2 days produced cells that could respond to stimuli. This basic study showed that i) cells did not detach from the matrix, ii) cells had a decreased proliferation rate after 2 days culture in limited nutrients, and iii) cells recovered from the limited nutrient period and show proliferation both by thymidine incorporation and by microscopically determining the number of viable cells. The procedure can be summarized as follows:

   a) Day 1: Plate cells at a density which will permit them to remain in culture 1 week (10,000 /2 sq. cm. Plate in complete medium containing 7% fetal bovine serum and other usual additives. (Serum is necessary for the cells to attach to the plastic dishes).

   b) Day 3: Remove the fluid, gently wash the cultures with saline. Replace with serum-free medium containing NO additives.

   c) Day 4: Add agonist/antagonists to study generation of bioactive lipids, alterations in phosphorylation patterns, cross talk among regulatory pathways, etc.

2. **Description of proliferation assays to assess inhibition of cellular growth.** Cells were plated in 96 well cluster plates day 1, drug was added day 2, and on day 4 the experiment was ended by removal of culture fluid, placing the plate in the freezer (to disrupt cells). A lysis buffer was added to wells to further disrupt the cells and a ligand which emits fluorescence when bound to DNA was incubated for 10 min. Fluorescence was determined by using a Fluorescence detection plate reader.

3. **Detection of heteropoly anion (HPA) free-radical scavengers in tissues:** The mice administered HPA compounds were euthanized by CO2 and the organs examined and removed. Each organ was digested in 1M Nitric Acid, neutralized with 1 M NaOH and diluted. Tungsten was quantitatively determined by use of Atomic Absorption Spectral Analysis using ICP adaption. Syntheses of high oxidation state manganese-substituted heteropolyanions. We first isolated and characterized Mn^{IV} substituted Keggin polyanions ($[XW_{11}\ Mn^{IV}\ O_{40}\ ]^{n-}$, X = Si, B, and Zn) using X-ray diffraction, Extended X-ray absorption fine structure method, magnetic susceptibility, electrochemistry, and routine spectroscopic methods {35}. Di-manganese substituted γ-Keggin polytungstosilicates, γ-$[SiW_{10}\ Mn_2\ O_{40}\ ]^{m-}$, were synthesized{22-25, 29-34}. Oxidation of tetra-manganese substituted polyaions, $[P_2\ W_{18}\ Mn_4\ O_{68}\ \bullet 2H_2\ O]^{10-}$ game two mixed valent compounds were synthesized and characterized {28} as previously described {35-38}. Oxidation of alkylene with iodosobenzene (PhIO) was examined using above mono-, di-, and tetra manganese-substituted polyanions in the presence and absence of air. The oxidized products were identified by GC-Mass and comparison made of the retention time with authentic compounds.
4. **Arachidonate metabolism**: HPLC separation, identification and quantitation of ca. 30 arachidonate metabolites in a single run was devised by our laboratory {27}. We have established a pattern for determination of the kinetics of formation of even transient arachidonate metabolites in cell cultures +/- agonists or antagonists. The method can be adapted for the separation, and identification of radioactive metabolites from cell cultures, or for separating, identifiying and quantitating minuscule amounts (fMol & pMol) in samples from animals or tissues.
5. **Agonist stimulated arachidonate metabolism**: IGF-I stimulates proliferation quiescent MCF-7 cultures {43}. The objective of this proposal is to identify bioactive lipids which are being generated in response to growth factor stimulation in the presence and absence of specific arachidonate inhibitors or heteropolyanion treated MCF-7 cells. The MCF-7 cell cultures (an adherent cell line) will be plated in 4-well cluster plates and 24 hr later will be incubated overnight arachidonic acid (approximately 10 uCi/well). We have found this culture pattern to produce metabolites with sufficient radioactivity. The culture fluid will be removed, a saline wash performed, and buffer added (buffer designed for optimal phospholipase A2 activity). Control and agonist/antagonists will be added and the reaction stopped (acidified) at selected time periods from 15 sec through 2 hr (about 12 different time points for each series (control, agonist, etc.). We usually limit an experiment to approximately 50 wells. The cells will be scraped from the wells, briefly sonicated to insure cell disruption (some arachidonate metabolites may remain intracellular), and centrifuged at 20,000 x g to pellet all insoluble material. The internal standard is then added to the supernatant solution, the pH adjusted, arachidonate metabolites extracted and the samples prepared and run on reverse phase using a C-18 column for HPLC analysis. Platelet activating factor fraction will be separated away from arachidonate metabolites at the extraction step and will be assayed. Meanwhile, the pelleted material will be extracted by Bligh-Dyer procedure and the extracts applied to thin layer plates to run in solvent systems designed to resolve phospholipids or diglycerides/fatty acids. The latter techniques give interesting information when comparing

phospholipids present at the beginning of the experiment (samples under 2-3 minutes) with those present at the end of the experiment (>2 hr).

9. Toxicity testing in mice: HPA-SM was administered to Balb C, 6-8 week old mice using a 12 gauge needle inserted subcutaneously under the loose skin on the back of the mouse. HPA-NA (0.2 mg)was administered once weekly for 10 weeks, 0.5 for 7 weeks and 0.7 for 3 weeks. At the end of the time periods, the mice were sacrificed, extensive necropsy was performed and all organs were examined. No toxicity or abnormalities were observed. Plasma levels of drug were determined using atomic absorption spectrometry.

RESULTS AND DISCUSSION:

1. Manuscript #1 (in final stages of completion for submission to *Journal of Clinical Investigation*), Appendix. In this study, 3 ER-positive and 3 ER-negative breast cancer cell lines were examined for mRNA expression for 5-LO, COX-1 and COX-2 transcripts. These analyses demonstrate that all cell lines tested uniformly express mRNA for 5-LO, FLAP, LTC-4 synthase and COX-1. COX-2 was expressed in 4/5 cell lines. LO enzymes producing 12 & 15 modified eicosanoids were expressed in 3/6 and 5/6, cell lines respectively. Clinical specimens of malignant and normal breast tissue express mRNA for 5-LO. Exogenously added growth factors (transferrin and IGF-I) stimulated the synthesis of 5-HETE (a 5-LO product). Addition of 5-HETE to breast cancer cell cultures directly stimulated growth (>25%). Inhibition of 5-LO metabolism by selective antagonists resulted in growth reduction of the tumor cell lines tested. Inhibitors of the FLAP were most potent. In contrast, cyclooxygenase inhibitors at concentrations up to 100 uM were not toxic and were frequently stimulatory. Breast cancer cells exposed to 5-LO inhibitors demonstrated increased frequency of apoptosis; this is consistent with the mechanism for the anti-proliferative effect of the inhibitors being mediated via up regulation of apoptotic growth control.
2. Manuscript #2 (in final stages of completion for submission to *Cancer Research*), Appendix. In this study, the attempt was to examine many more classes of inhibitors of eicosanoid metabolism. A pair of cell lines were chosen, the parent ER-positive MCF-7 and the multidrug resistant, ER-negative daughter clone MCF-7 ADR[10]. Figure 1 of the report (not the manuscript), shows a simplified schematic diagram of eicosanoid metabolism. The object of this study was to examine more fully the effects of blocking other key pathways in bioactive lipid generation. Among the classes of inhibitors examined were nonspecific LO inhibitors, a number of 5-LO or FLAP inhibitors, blockers of cytochome P-450 eicosanoid formation, PKC inhibitors and drugs which inhibit PAF formation. These findings show that targeting these lipid metabolism pathways is equally effective for drug-resistant and sensitive cells and is independent of ER status. We observed that the non-specific LO drugs caused accumulation of cells in Go/G1 phase and then led to apoptosis. In contrast, the 5-LO blocking drugs slowed the synchronized culture progression through the cell cycle, finally accumulating cells in G2/S prior to impending apoptosis. We further examined the most successful of these inhibitors in cultures of human bone marrow cells and found

several of them to exhibit little toxicity to the bone marrow cultures. The thorough examination of the bone marrow cultures included evaluation of various types of progenitor cells (table 1, report). Those data show that at effective concentrations of drugs, only CFU-E cells were affected. Even at lowest doses of drug, those cells were the most sensitive. However, the two lowest doses of drug did not reduce CFU-E below acceptable levels. These data, taken as a whole, suggest that these lipid metabolism pathways may be critical to survival of breast cancer cells and can be successfully targeted to control the rampant proliferation typical of breast cancer.

3. Manuscript #3 (under preparation for submission), Appendix. We synthesized a series of heteropolyanion drugs for targeting metabolism of breast cancer cells. Of these drugs, we selected 2 relatively non-toxic compounds for further studies. These drugs did not induced multidrug resistance, were equally effective in blocking proliferation and inducing apoptosis in drug resistant and sensitive cells and independent of ER-status. The drugs exhibit relative lack of toxicity in bone marrow cultures and in mice. These drugs have the capacity to be free-radical scavengers which means that they should block utilization of arachidonic acid and its subsequent metabolism. Preliminary data (this report, Fig 2), suggests a role in altering production of bioactive lipid intermediates. We are proceeding to evaluate the data for possible pursuit of IND approval.
4. Manuscript #4, Appendix (final stages of completion for submission to the *Journal of Biological Chemistry*). This manuscript attempts to put in perspective the mechanistic regulatory constraints involving mitogen-activated protein kinases (MAPK), stress activated protein kinases , signal transducers and activators of transcription (STAT), janus kinase (JAK). The data in this manuscript demonstrate that 5-LO pathway regulation impinges on the kinase activation cascades. These findings have been presented at an international meeting (International Congress of Biochemistry, San Francisco, August 97) and have generated a great deal of interest because of the expansion to our knowledge of the regulatory mechanisms which they have revealed.
5. Additional information contained in graphs that are part of this report (in contrast being a part of one of the manuscripts).

   Arachidonate metabolism in response to IGF-I in the presence and absence of various inhibitors of eicosanoid metabolism in T47D breast cancer cells. In all of the following graphs of AA metabolites, the "fold change" (Y-axis) was calculated by dividing the value for "control" cultures (no additions) into the value for the indicated regimens. Therefore, any fold-change other than "1", indicates a change relative to control. When inhibitors were used, they were added 30 min prior the fluid change which occurred immediately before addition of IGF-I. It is important to determine which of these metabolites rapidly appear (2 min) and which persist (30 min).

   Figures 2-3, analyses of AA metabolites 2 min after agonist addition to cells shows several classes of metabolites elevated relative to control. This overview graph is used to identify, those metabolites showing the greatest changes relative to control values. IGF-I stimulated production of 5- and 15-HETE/HPETEs and their metabolites, methyl esters (ME) and δ-lactones (D LAC).

IGF-I elevated most of these metabolites about 2-fold over control values. Quite striking was the elevation of most metabolites by MK591, the FLAP inhibitor alone, with values ranging from 12-fold increases in LTE4, to mostly 6-8 fold increases in prostanoids, LTB4, and 15-HPETE. Addition of IGF-I to cultures pre-incubated with MK591, brought all metabolite levels down to base levels except LTE4 and LTB4. Nordiguaretic acid (NDGA) alone showed no significant changes relative to control; NDGA + IGF-I showed an accumulation of 5-HPETE (the parent compound of the 5-LO pathway) and appeared to arrest further metabolism of AA to produce the incredibly potent 5- , and 15-LO bioactive lipids. The same analysis is indicated for the pair HPA-Na alone and HPA-Na + IGF-I. In that case, as with NDGA, 5-HPETE was elevated; the HPA-Na pair also forced the accumulation of AA, apparently preventing its utilization.

Figures 4-5, show analyses of AA metabolites 30 min after agonist addition to cells. IGF-I stimulated persistence of 5-LO products (LTC4 & 5-HPETE) and 15-HETE. By this time period, all three drugs +IGF-I stimulated accumulation of massive increased amounts of 15-HETE ME. The HPA-Na +IGF-I induced the accumulation of LTC4 about 24-fold relative to control values. In general, the less biologically active molecules were not present and parent or branchpoint molecules accumulated (see Fig 1). These data suggest that 5-, and 15-LO pathways are massively utilized by breast cancer cells. There are few specific inhibitors available which target the 15-LO pathway.

WORK ACCOMPLISHED COMPARED TO STATEMENT OF THE WORK (in appendices):

1. Up to this point, we have completed all the work outlined to be accomplished in years 1, 2 and 3. We are now in the process of summarizing our data and publishing it in peer reviewed journals. The enclosed manuscripts, 3 of which are in the final stages of completion prior to submission and the 4th is being revised (Dr. Zhang, a suberb scientist, has some expressions which are not standard English for a formal manuscript).

FUTURE DIRECTIONS AND GOALS:

1. One of our most urgent objectives is to identify if a correlation exists between 5, 15-HETEs, their metabolites, etc and cellular proliferation. We have a wealth of data to aid us in identifying inhibitors that could interfere in these pathways. Identify and use of combinations of selected drugs for evaluation in breast cancer models.
2. Select the most promising drugs and combinations; test in an in vivo model of breast cancer in mice.
3. Complete manuscripts in preparation and submit to peer reviewed journals; determine if HPA-NA drugs offer sufficient promise to initiate IND procedures. Summarize all accumulated data.

PROBLEMS ENCOUNTERED:

No untoward problems were encountered this year. We would like to take this opportunity to say that the "Era of Hope" breast cancer review meeting was especially beneficial this year.

ACCOMPLISHMENTS:

1. We are exploring the possibility of IND evaluation for our newly synthesized HPA drugs such as HPA-NA or HPA-Sm.

MANUSCRIPTS:

1. Reduced proliferation of Breast Cancer by Interruption of the 5-Lipoxygenase Pathway of Arachidonic Acid Metabolism. Avis, Hong, Vos, Martinez, Moody, Jett and Mulshine. To be submitted to J. Clin. Investigation.
2. Control of Growth of Human Breast Cancer Cells by manipulation of Arachidonate Metabolism. To be submitted to Cancer Research.
3. Heteropolyanion Free-Radical Scavengers: Induction of Apoptosis in Human Breast Cancer Cells. Manuscript in preparation.
4. Cross Talk Between Map Kinase Pathway And Arachidonic Acid Pathway In The Signaling Cascade Of Igf-1 In Breast Cancer Cells. R. Das, N. Kodsi and M. Jett. To be submitted to J. Biol Chem.

ABSTRACTS:

1. Das, R., Kodsi, N. and Jett, M. (1997) Cross talk between the MAP kinase and the arachidonic acid pathway in signal transduction of growth factor in breast cancer cells. FASEB Journal 10: 2852.

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(7) CONCLUSIONS

a) We have shown, using 3 ER-positive and 3-ER negative breast cancer cell lines 5-LO and COX-1 mRNA espression in all. COX-2, 12-HETE, and 15-HETE were also expressed in most of that battery of cells. IGF-I and transferrin each stimulated production of 5-HETE in 4 different breast cancer cell lines. Furthermore, inhibition of FLAP induced apoptosis in breast cancer cells.
b) We identified that blocking other lipid metabolism enzymes caused cells to accumulate in Go/G1 phase of the cell cycle and subsequently induced apoptosis. A full array of these drugs were evaluated for toxicity to bone marrow cells and many were found to exhibit minor toxicity.
c) Synthesis of HPA drugs provided another class of bioactive lipid targeting inhibitors. These water soluble, stable drugs effectively blocked proliferation in breast cancer cultures yet showed little toxicity in bone marrow cultures. Mice were given regimens for 3-10 weeks of the HPA-Na drug; necropsy revealed no toxicity other than hair loss at the site of drug administration. We are evaluating the option of pursuing IND approval.
d) We have identified cross talk between MAP kinase pathway and arachidonic acid metabolism stimulated by IGF-I in breast cancer cells. These important mechanistic studies open up new avenues for selection of possible drug treatments and regimens to combat breast cancer.

e) Arachidonate metabolism was analyzed in T47D (ER-positive) breast cancer cells. The studies show the LO pathway to be critical for breast cancer proliferation. Of the drugs used in this study, all block production of biologically active forms of the metabolites and cause accumulation of parent compounds in the arachidonic acid cascade.

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(9) APPENDICES

ABBREVIATIONS / GLOSSARY

AA = arachidonic acid or eicosatetraenoic acid, the parent eicosanoid compound.
LA= linoleic acid, a precursor molecule for biosynthesis of arachidonic acid
LO = lipoxygenase, one of two major series of enzymes metabolizing AA (See Fig 1).
HETE = hydroxyeicosatetraenoic acid (5-, 12-, 15-); The number associated with these metabolites indicate the carbon atom on which a hydroxyl has been added.
HPETE=hydroperoxyeicosatetraenoic acid; peroxy group added to AA at Carbon#.
LT=leukotrienes designated A,B,C,D,E4 (i.e., LTD4). Potent 5-LO metabolites with a wide range of biological activities.
Lipoxins= di- and tri-HETEs. Metabolites formed by hydroxylation of multiple carbon atoms of AA. Potent biological derivatives which stimulate protein kinase C
EA=eicosapentanoic acid; a synthetic eicosanoid which does not occur in nature. We have synthesized it for use it as an internal standard in our analyses of AA metabolism.
COX-I= The constitutive cyclooxygenase necessary for proper cellular function;
COX-2= The inducible cyclooxygenase, known to be responsible for pain and inflammation; may have other activities as yet undescribed
PLA2 = phospholipase A2, the enzyme which releases AA from phospholipids
FLAP = 5-lipoxygenase activating protein
PG= prostaglandins, such as PGE2= prostaglandin E2; PGF2, etc.
TBX=thromboxanes
EGF= epidermal growth factor;
IGF-I= insulin-like growth factor-I
JNK= Jun Kinase;
MAPK= Mitogen activated protein kinase
STAT=Signal transducers and activators of transcription
PPAR = perixosome proliferator-activated receptor
RXR = Retinoic X Receptor; RAR = Retinoic Acid Receptor
HPA's = heteropolyanions . A list of HPA's and an example of the structure are shown in manuscript #3.
FABP = fatty acid binding proteins; L-FABP= liver type; I-FABP= intestinal type; H- FABP = heart type; MDGI = mammary derived growth

inhibitor, a heart-type FABP; CRABP= Cellular Retinoic Acid Binding Protein
PKC=protein kinase C

LEGENDS TO FIGURES

Figure 1. A schematic diagram showing some of the major bioactive lipid metabolites of arachidonic acid. Pathway A indicates non-specific lipoxygenases; B, 5-lipoxygenases, C, peptidoleukotrienes; and D, cyclo-oxygenases.

Figure 2-5. Arachidonic acid metabolites produced in response to IGF-I in the presence and absence of various drugs that target lipid metabolism. In all of the following graphs of AA metabolites, the "fold change" (Y-axis) was calculated by dividing the value for "control" cultures (no additions) into the value for the indicated regimens. Therefore, any fold-change other than "1", indicates a change relative to control. When inhibitors were used, they were added 30 min prior the fluid change which occurred immediately before addition of IGF-I.

Figures 2-3. Analysis at 2 min post IGF-I for "5-LO products" (Fig. 2) and "Prostanoids & 12-, 15-LO products (Fig. 3).

Figures 4-5. Analysis at 30 min post IGF-I for "5-LO products" (Fig. 4) and "Prostanoids & 12-, 15-LO products (Fig. 5).

FIGURE 1

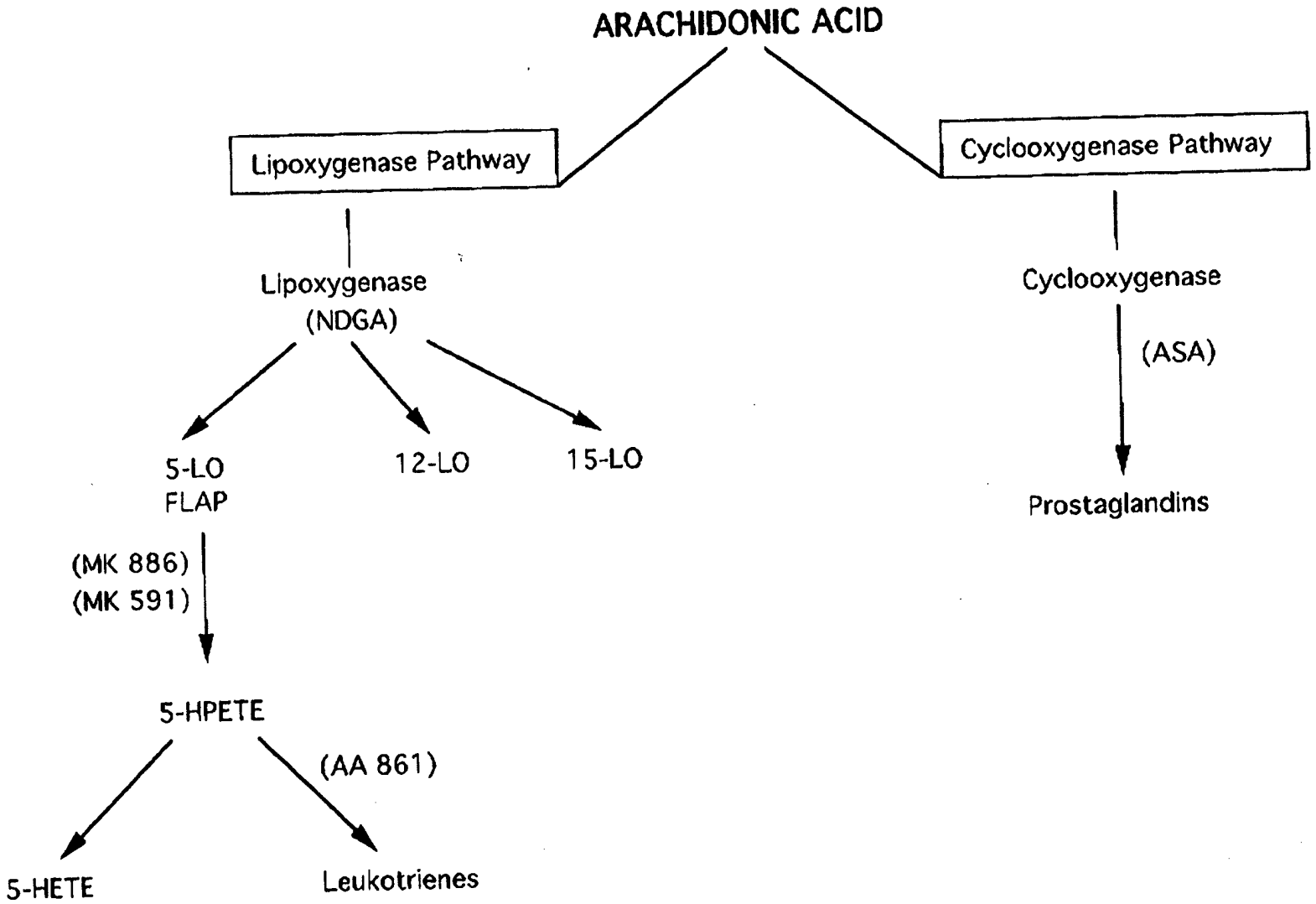
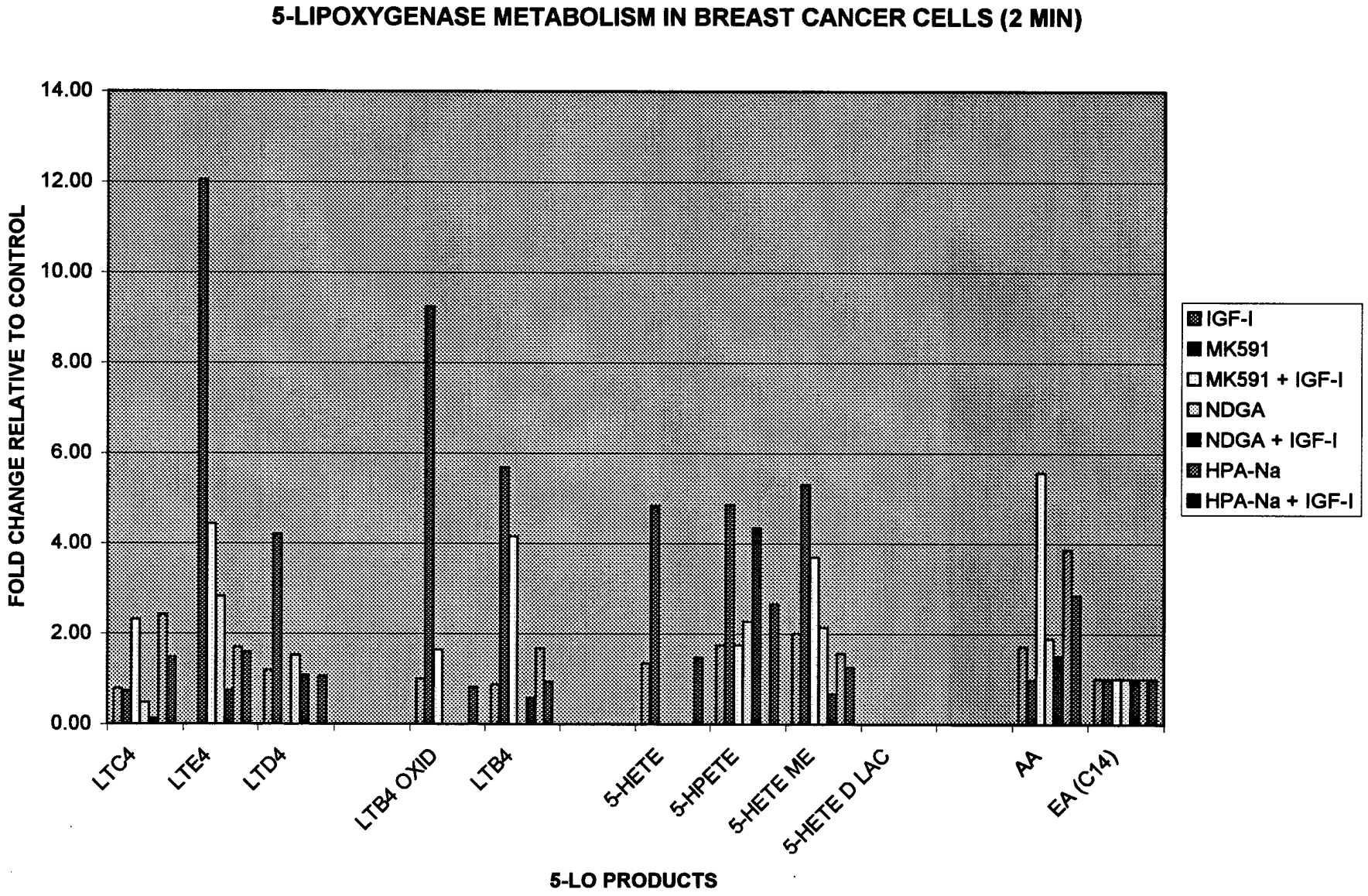


FIGURE 2


5-LIPOXYGENASE METABOLISM IN BREAST CANCER CELLS (2 MIN)
FOLD CHANGE RELATIVE TO CONTROL
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
LTC4
LTE4
LTD4
LTB4 OXID
LTB4
5-HETE
5-HPETE
5-HETE ME
5-HETE D LAC
AA
EA (C14)
5-LO PRODUCTS
IGF-I
MK591
MK591 + IGF-I
NDGA
NDGA + IGF-I
HPA-Na
HPA-Na + IGF-I

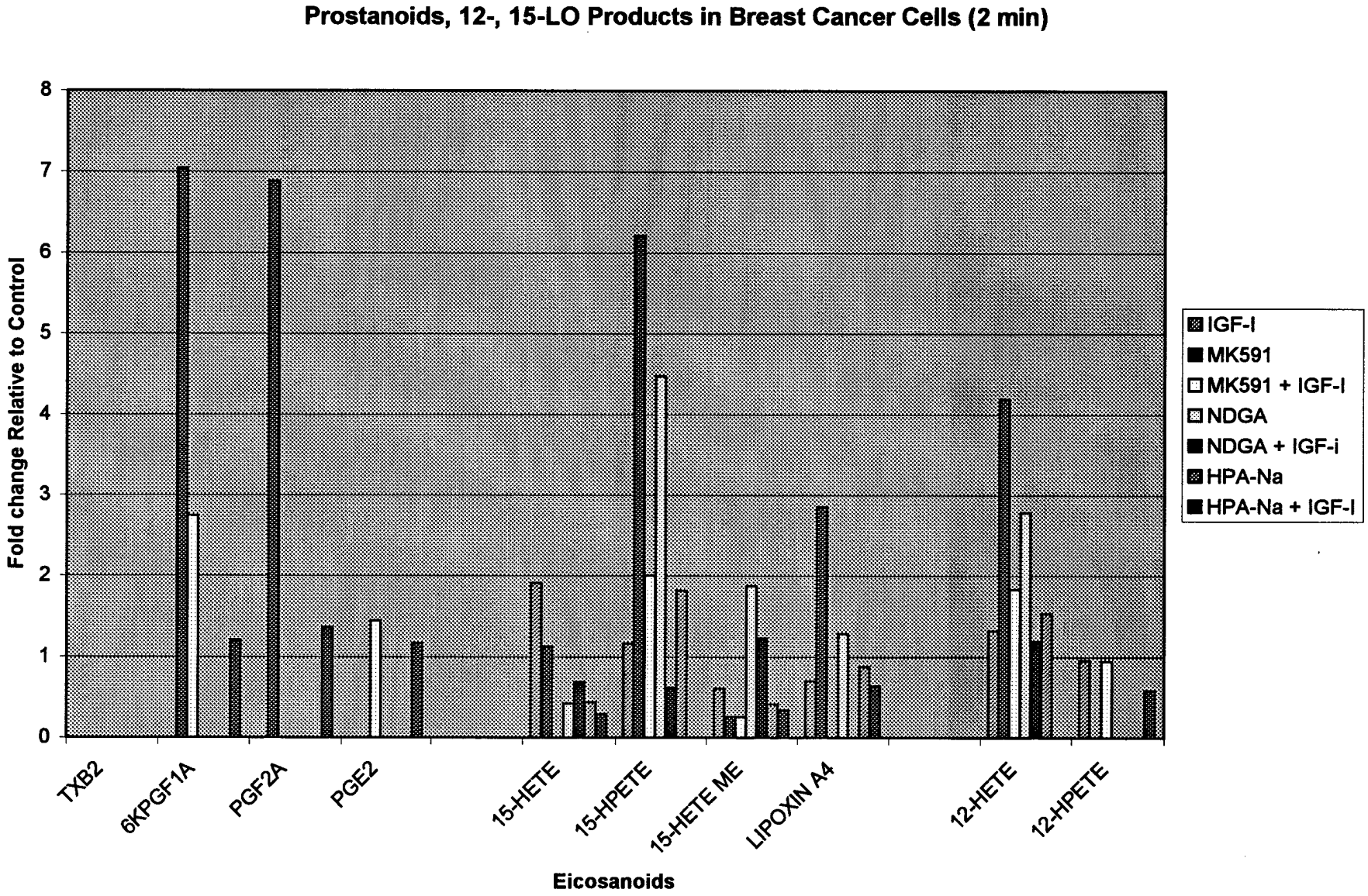

Prostanoids, 12-, 15-LO Products in Breast Cancer Cells (2 min)
Fold change Relative to Control
0
1
2
3
4
5
6
7
8
TXB2
6KPGF1A
PGF2A
PGE2
15-HETE
15-HPETE
15-HETE ME
LIPOXIN A4
12-HETE
12-HPETE
Eicosanoids
IGF-I
MK591
MK591 + IGF-I
NDGA
NDGA + IGF-i
HPA-Na
HPA-Na + IGF-I

FIGURE 4

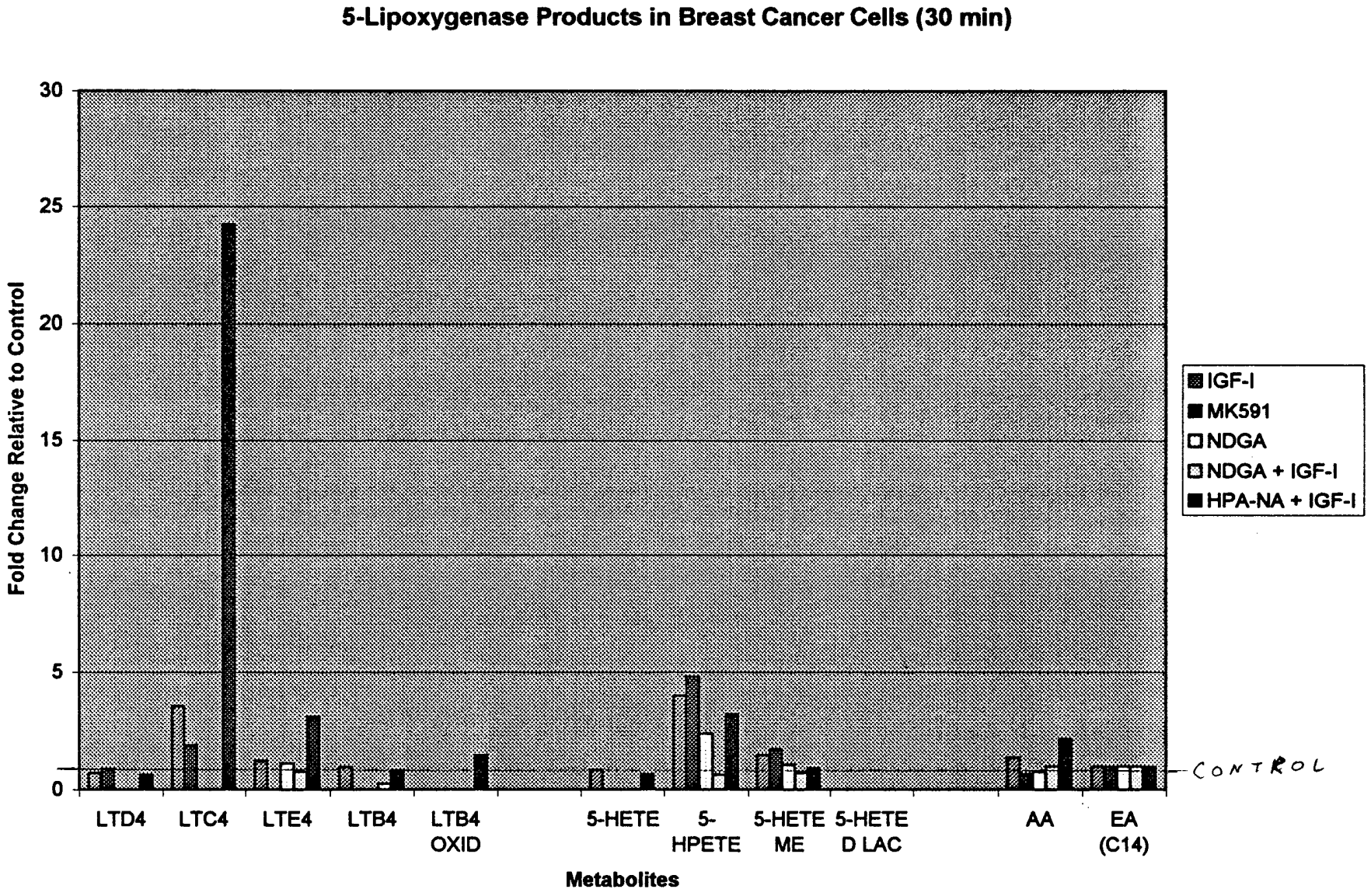
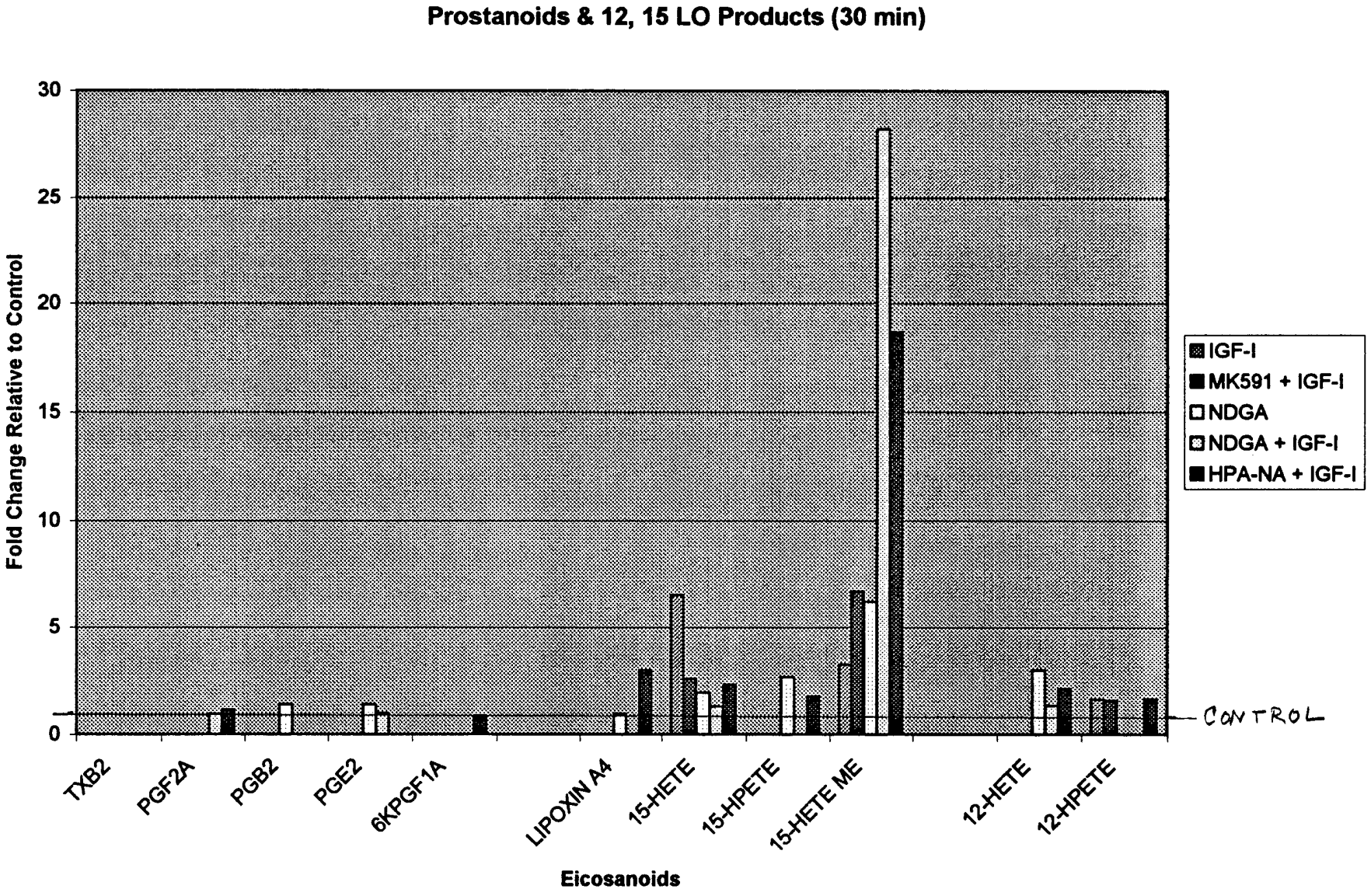

5-Lipoxygenase Products in Breast Cancer Cells (30 min)
Fold Change Relative to Control
30
25
20
15
10
5
0
IGF-I
MK591
NDGA
NDGA + IGF-I
HPA-NA + IGF-I
CONTROL
LTD4
LTC4
LTE4
LTB4
LTB4 OXID
5-HETE
5-HPETE
5-HETE ME
5-HETE D LAC
AA
EA (C14)
Metabolites

FIGURE 5


Prostanoids & 12, 15 LO Products (30 min)
Fold Change Relative to Control
30
25
20
15
10
5
0
IGF-I
MK591 + IGF-I
NDGA
NDGA + IGF-I
HPA-NA + IGF-I
CONTROL
TXB2
PGF2A
PGB2
PGE2
6KPGF1A
LIPOXIN A4
15-HETE
15-HPETE
15-HETE ME
12-HETE
12-HPETE
Eicosanoids

ATTACHED MANUSCRIPTS

1. Reduced Prolferation of Breast Cancer by Interruption of the 5-Lipoxygenase Pathway of Arachidonic Acid Metabolism. Avis, Hong, Vos, Martinez, Moody, Jett and Mulshine. To be submitted to J. Clin. Investigation.
2. Control of Growth of Human Breast Cancer Cells by manipulation of Arachidonate Metabolism. To be submitted to Cancer Research.
3. Heteropolyanion Free-Radical Scavengers: Induction of Apoptosis in Human Breast Cancer Cells. Manuscript in preparation.
4. Cross Talk Between Map Kinase Pathway And Arachidonic Acid Pathway In The Signaling Cascade Of Igf-1 In Breast Cancer Cells. R. Das, N. Kodsi and M. Jett. To be submitted to J. Biol Chem.

Reduced Proliferation of Breast Cancer by Interruption of the 5-Lipoxygenase Pathway of Arachidonic Acid Metabolism.

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Abstract

Arachidonic acid metabolism has an important function in promoting the growth of cancer cells. Previously, we demonstrated that the lipoxygenase pathway of arachidonic acid metabolism is important in the growth regulation of lung cancer, we now report a similar role for this pathway in breast cancer. In this study, six breast cancer cell lines were analyzed for mRNA expression for lipoxygenase and cyclooxygenase transcripts. These analyses demonstrate that all cell lines tested uniformly express mRNA for 5-lipoxygenase, 5-lipoxygenase activating protein, LTC-4 synthase, and cyclooxygenase-1. In contrast cyclooxygenase-2 was expressed in 4/6 cell lines, 12, and 15-lipoxygenase were expressed in 3/6, and 5/6, cell lines respectively. We also show evidence that clinical specimens of malignant and normal breast tissue express mRNA for 5-lipoxygenase. Two exogenously added autocrine growth factors, insulin-like growth factor-1 and transferrin, induced the biosynthesis of the 5-lipoxygenase metabolite 5-HETE. Using a proliferation growth assay, our results demonstrate that 5-HETE can directly stimulate growth (>25% over control at 0.01 ug/ml) *in vitro*. Furthermore, inhibition of lipoxygenase metabolism by selective antagonists at 1-10 uM concentrations resulted in significant, reproducible growth reduction of 20-90% *in vitro* of the tumor cell lines tested. Inhibitors of the 5-lipoxygenase activating protein were most potent. In contrast, cyclooxygenase inhibitors at concentrations <100 uM had no significant effect. Breast cancer cells exposed to lipoxygenase inhibitors

demonstrate increased frequency of apoptosis, which is consistent with the mechanism for the anti-proliferative effect of the inhibitors being mediated via up regulation of apoptotic growth control. These results suggest that inhibitors of the lipoxygenase pathway may provide a new pharmacological method to reduce the growth of breast cancer cells.

Introduction

Arachidonic acid (AA) biosynthesis which is initiated after trauma, infection and inflammation, has functions that can promote a variety of carcinogenesis-related events [Steiner, 1994 #495]. The incidence of breast cancer in American women is approximately one in eight [Boring, 1993 #521], with a variety of metabolic and hormonal factors postulated as having a mitogenic effect in this disease process [Wynder, 1986 #522; Yano, 1992 #198; Martin, 1995 #482]. The clonal expansion of an early transformed epithelial cell and the progression of an advanced cell to metastatic disease are both mediated by chronic growth factor stimulation via activation of signal transduction mechanisms. Signal transduction pathways shared by different autocrine growth factors and/or oncogenes may therefore provide a more efficient approach to enabling meaningful control of breast cancer growth.

In a recent study with lung cancer cell lines, we have shown that the activation of the Type I, insulin-like growth factor receptor (IGF-R) involved the oxidation of AA, and that this pathway is central to growth stimulation. In that study, we demonstrated that IGF-R mediated growth stimulation can be neutralized by blocking the 5-lipoxygenase (5-LO) pathway of AA metabolism in lung cancer [Avis, 1996 #428]. We have previously confirmed that IGF-R expression is a conserved feature for breast, and lung cancer [Quinn, 1996 #497], and IGF-R has been proposed as playing a crucial role in malignant transformation [Baserga, 1994 #441]. Consequently, activation of IGF-R may have an important role in protecting

cells from programmed cell death, allowing the cancer cells to progress to the lethal invasive phase of carcinogenesis. Apoptosis is an important regulatory function in growth and development [Williams, 1991 #514; Ellis, 1991 #501], growth factors, and intracellular mediators of signal transduction, i.e. survival factors, have been shown to have physiological roles in the inhibition of apoptosis [Thompson, 1995 #436; Piazza, 1995 #437; Wang, 1995 #468]. Baserga et al., demonstrated that IGF-R activation by its ligand is important in the preservation of the transformed phenotype, and inhibits apoptosis [Baserga, 1994 #441]. Conversely, when the number of IGF-R were reduced using antisense strategies, massive apoptosis of tumor cells occurred, coincident with loss of tumorigenecity *in vivo* [Long, 1995 #506; Resnicoff, 1995 #450]. Recently, Tang et al., reported that AA lipoxygenase pathways functions as an important regulator of cell survival and apoptosis in rat Walker (W256) carcinosarcoma cells [Tang, 1996 #510]. Although the mecahnism is not clear, our previous study with lung cancer cell lines, suggest that the interruption of 5-LO cell signaling initiated by two autocrine growth factors (IGF-1, and gastrin-releasing peptide, (GRP)), up-regulates the rate of apoptosis [Avis, 1996 #428].

AA metabolism has been proposed to play a significant role in mammary carcinogenesis, indicating that modulation of various pathways could result in suppression of tumor growth [Earashi, 1995 #472; Noguchi, 1995 #474]. AA can be metabolized either by the cycloxygenase (COX) or the lipoxygenase (LO) pathways, and the genes for these enzymes have been cloned [Dixon, 1988 #517; Smith, 1996 #518]. A number of

pharmacological antagonists for the AA pathways are available (Figure 1), some of which are presently being used in specific clinical settings, with limited side effects [Henderson, 1994 #421; Giovannucci, 1995 #448]. Biologically active products of the 5-LO pathway include 5(S)-hydrooxyeicosa-6E,8Z,11Z,14Z-tetraenoic acid (5-HETE) and leukotrienes, which are thought to be central to the pathopysiology of a variety of diseases, including asthma. The regulation of these products can be achieved either by direct inhibition of 5-LO, or indirectly through the action of inhibitors binding with high affinity to 5-LO activating protein (FLAP) [Ford-Hutchinson, 1994 #511]. FLAP and leukotriene C4 synthase have been shown to have significant sequence identities, representing alternative targets to inhibit leukotriene biosynthesis [Ford-Hutchinson, 1994 #520; Jacobsson, 1996 #519]. In the present study, we report that inhibitors of the 5-LO pathway are effective in reducing the *in vitro* growth of breast cancer cell lines. Our *in vitro* and *in vivo* studies suggest that the interruption of 5-LO activation may leave the breast cancer cells vulnerable to apoptotic clearance, and therefore may play an important role in tumor cell regulation. Our data further suggest that 5-LO inhibitors may have efficacy as a clinical tool for control of breast cancer.

Materials and Methods

Cell lines: Cell lines used in the study were obtained from the American Type Culture Collection, (Rockville, MD) and Dr. Powel Brown (BPRB, NIH), They included three estrogen-receptor (ER) positive cell lines MCF-7 wild type (MCF-7 WT), ZR-75, and T47 D. Three ER- negative cell lines, SKBR-3, MB-231, and MCF-7 adriomycin resistant (MCF-7-ADR), were also included in the study. The cells were maintained in RPMI-1640, or MEM Zinc option medium (Richter's Modification), supplemented with 5% fetal bovine serum (FBS), penicillin (50 units/ml) and streptomycin (50 ug/ml) (Gibco, Grand Island, NY), in a humidified atmosphere of 95% air and 5% CO_2 atmosphere at 37^oC.

Chemicals: Synthetic 5-HETE was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Lipoxygenase inhibitors nordihydroguaiaretic acid (NDGA), 2(12-hydroxydodeca-5-10-dinyl)-3,5,6-trimethyl-1-4-benzoquinone inhibitor (AA861), and FLAP inhibitor, MK 886 (3-[1-(p-chlorobenzyl)-5-(isopropyl)-3-tert-butylthioindol-2,2-dimethylpropanoic acid), were also obtained from BIOMOL Research Laboratories. The structurally novel FLAP antagonist MK-591 (3-[1-(4-chlorobenzyl)-3-(t-butyl-thio)-5(quinolin-2-yl-methoxy)-indol-2,2-dimethyl propanoic acid) was a kind gift from Merck Frosst Centre for Therapeutic Research (Pointe Claire-Dorval, Quebec, Canada). The cyclooxygenase inhibitors acetylsalicylic acid (ASA), and indomethacin was

purchased from Sigma Chemicals (St. Louis, MO). Antibodies to the *bcl-2*, and *bax* genes were purchased from Oncogene Research Products (Cambridge, MA).

Message expression: mRNA expression for *LO and COX* transcripts was determined using the reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA (5ug) was isolated from each cell line, DNAse treated, and reverse transcribed into cDNA, using the Superscript II RNAse H^- Reverse Transcriptase Systems (GibcoBRL). One ul of the cDNA reaction mix and 100 ng of gene specific oligonucleotide primers, selected using Lasergene Software by DNASTAR (Madison, WI), were used in the PCR. Forward (F) and reverse (R) primers used to detect 5-LO, FLAP, 12, and 15-LO cDNAs in the amplification were:

5LO-F (5'-CCCGGGGCATGGAGAGCA-3'),

5LO-R (5'-GCGGTCGGGCAGCGTGTC-3'),

12LO-F (5'-GGCCCGGACCCAACTCATCTC-3'),

12LO-R (5'-GCATTAGGGACCCAGGCATACCAG-3'),

15LO-F (5'-GCTGCGGCTCTGGGAAATCATCT-3'),

15LO-R (5'-GGGCCCGAAAAATACTCCTCCTCA-3'),

FLAP-F (5'-GCTGCGTTTGCTGGACTGATGTA-3'),

FLAP-R (5'-TAGAGGGGAGATGGTGGTGGAGAT-3').

The primers used to detect LTC4 synthase were:

LTC4-F (5'-CCA GCT CGC CTT CAC ACA CAG -3),

LTC4-R (5'-TTG CAG CAG GAC TCC CAG GAG -3).

mRNA expression for *COX-1*, and *COX-2*, was determined as described

above, and the primers used to detect COX-1 and COX-2 cDNAs in the amplification were:

COX1-F (5'-CACTCACGGGCGCTGGTTCTGG-3'),

COX1-R (5'-CTGGCTCTGGGGCGGGATGC-3'),

COX2-F (5'-TGTGGGGCAGGAGGTCTTTGGTCT-3'),

COX2-R (5'-GCATCTGGCCGAGGCTTTTCTAC-3').

Hot start PCR reactions were performed for 35 cycles with 94°C denaturation for 15 seconds, 60°C annealing for 15 seconds, and 72°C extension for 1 minute. RT-PCR products were fractionated on a 1.2% agarose gel and Southern blotted onto nitrocellulose. Southern blots were hybridized to radiolabelled internal primer probes for each gene. Amplification products for both 5-LO, FLAP, 12-LO, and 15-LO were of the expected size as determined by ethidium bromide staining (data not shown), and Southern blot analysis.

In situ RT-PCR was performed following established methods [Martinez, 1995 #439] with the same 5-LO primers used as above. Nine normal and nine malignant breast tissue specimens were kindly provided by Dr.-----for these analysis. Briefly, after proteinase K digestion the sections were subjected to reverse transcription with the same kit used for regular RT-PCR. The PCR mixture (Perkin Elmer Cetus, Norwalk, CT) contained 2.5 mM $MgCl_2$, 2.5 U/100 ul Taq DNA polymerase, 200 mM dNTPs, 100 uM digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN), 1 ng/ml primers, 50 mM KCl, 10 mM Tris-HCl, pH 8.3. To achieve a synchronized "hot start", Taq polymerase was blocked with TaqStart antibody (Clontech). The incorporated digoxigenin was localized with the

Digoxigenin Detection Kit (Boehringer Mannheim, Indianapolis, IN).

Radioimmunoassay (RIA) analysis for the production of 5-HETE: The samples were prepared and extracted as previously described [Boyle, 1994 #275; Avis, 1996 #428]. Briefly, after exposure to the growth factors Transferrin (TF); and IGF-I (10 ug/ml and 5ug/ml respectively), the cells were disrupted and extracted onto C-18 disposable cartridges. The eicosanoids were eluted using 85% acetonitrile/15% MeOH. The samples were dried by rotary vacuum evaporation, reconstituted in ethanol, and diluted in RIA buffer provided with the RIA kit. The RIA was assayed for the appropriate metabolite in duplicates according to manufacturers protocol, and the values obtained averaged. The RIA kit does not distinguish 5-HETE from its derivatives d lactone and methyl ester.

Growth studies: We used a modification (Promega CellTiter 96^{TM}), (Promega, Madison, WI) of the semiautomated MTT colorimetric assay [Nakanishi, 1988 #28]which quantitates cell numbers based on the reduction of a tetrazolium compound by tumor cells to a colored formazan end product, which is quantitated by measuring the change in optical density (570 nm) compared with a control. Seeding densities were 1-$2x10^4$ cells/well, and cells were grown for 5 days. Experimental conditions were as previously described [Avis, 1996 #428]. Experiments were repeated at least three times.

In vitro apoptosis analysis: The cells were treated *in vitro* with

antagonists in 8 well cytochambers at comparable cell and drug concentrations used in the growth assay. The cells were washed in PBS and fixed in 95% ethanol and stored frozen until used. In some experiments cytospins were used instead of cytochambers. The cell lines were analyzed for the presence of apoptosis-induced nucleosomes using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD), following manufactures instructions. The number of apoptotic cells was counted in sets of one hundred cells (40X magnification) of each experimental well, and the percentage of apoptotic cells was determined. Similarily, immunohistocchemical (IHC) analysis for *bcl-2*, and *bax* expression was performed on cytospins of MCF-7 cells, using a Zymed Histostain kit (Zymed Laboratories Inc., San-Francisco, CA).

In vivo apoptosis analysis: MCF-7 tumor cells (10^7/mouse) were subcutaneously injected into the flanks of athymic nu/nu Balb/c mice, and a palpable mass formed after 7 days. Treatment begun on day 7 and consisted of 3 groups of five mice each. One group of 5 mice were given MK 591, initial dose 2.5 mg/day s.c., for the first week, this dose was reduced 3-fold for the remainder of the study due to skin toxicity. A second group of mice were given 0.1 % NDGA in their drinking water; the third group (placebo group), received 100 ul of PBS (s.c.) daily. The duration of the study was four weeks, and the tumors were measured biweekly. For the apoptosis experiments, tumors from each of the MK 591, NDGA, and placebo groups were harvested, fixed in 10% buffered formalin for 24 hours and paraffin-embedded. The tissue sections were analyzed

for the presence of apoptosis-induced nucleosomes using the ApopTag in situ apoptosis detection kit. The number of apoptotic cells was counted in ten microscopic fields (40X magnification) of each case.

Statistics: Significance of difference between samples was determined using Student's paired *t* test. $p<0.05$ was regarded as significant.

Results

mRNA expression:

To confirm that breast cancer cell lines express the enzymes which are the targets of the lipoxygenase inhibitors used in the study, we analyzed the mRNA expression for both 5-LO and FLAP using RT-PCR analysis. Figure 2 shows that all six cell lines tested express transcripts for both enzymes. To further define that these results were not *in vitro* artifacts, we also used *in situ* RT-PCR. This analysis revealed the expression of 5-LO mRNA in paraffin sections taken from nine normal breast tissue and malignant tumors. A representative example is shown in Figure 3. Of interest is that strong expression of 5-LO could also be found in morphologically normal breast tissue (9/9). To explore if other LO enzymes, as well as COX enzymes were expressed in the breast cancer cell lines, we evaluated the mRNA expression for LTC4, 12, and 15-LO, as well as COX. A summary of the mRNA expression of all enzymes analyzed is shown in Table 1. In contrast to the uniform expression of 5-LO and FLAP, 3/6 cell lines expressed 12-LO mRNA, and 5/6 cell lines expressed 15-LO mRNA. In our study, COX-1 was uniformly expressed, and COX-2 was expressed in 4/6 cell lines analyzed. LTC4-synthase was also uniformly expressed in all cell lines tested. From the small number of cell lines evaluated, expression of LO enzymes and FLAP, as well as COX enzymes and LTC4-synthase preliminarily appears to be unrelated to ER status for these cell lines.

Endogenous production of 5-HETE and its byproducts in response to growth factors

Lipoxygenase rapidly metabolize AA to hydroperoxy-eicosatetetraenoic acid, which subsequently gets metabolzed to 5-HETE and its derivatives. To demonstrate the presence of bioactive 5-LO enzyme in breast tumor cells, we utilized a specific RIA for 5-HETE. In each of the four breast cancer cell lines, stimulation with IGF-I or TF increased the production of 5-HETEs 2-4 fold above control levels (Figure 4). The IGF-I induced production of 5-HETE was modestly greater than TF in three cell lines.

Regulation of tumor cell growth:

To assess the relevance of 5-LO activity on breast cancer, we evaluated the growth stimulation of breast cancer after exogenous addition of its product, 5-HETE, to tumor cells using serum-free conditions in an *in vitro* proliferation assay. The data summarized in Table 2, demonstrates that the exogenous addition of 5-HETE will increase the tumor proliferation 25-50% above control in four of five breast tumor cell lines tested. A representative experiment is shown in Figure 5. A significant, reproducible, dose dependent increase in cell growth was observed, with the exogenous addition of 0.005-0.5 ug/ml 5(S)-HETE. Other related compounds such as 5(R)-HETE; 5±-HETE; or 5oxoETE had no significant proliferative effect on the cell lines tested (data not shown). In addition no consistent growth effects were observed using 12(S)-HETE or 15(S)-HETE

under the same experimental conditions (data not shown).

To determine the biological effect of AA metabolic pathway inhibitors on breast tumor cell line growth, we analyzed several different inhibitors on the growth of breast tumor cell lines. Table 3 summarizes the mean growth inhibition for each compound evaluated. Our analyses revealed a significant, potent and reproducible growth inhibition with 5 uM NDGA on all cell lines tested. With another direct 5-LO enzymatic inhibitor AA861, we observed growth inhibition to a lesser degree (3/6 cell lines), and a higher drug concentration (10 uM) was generally required with this compound.

We next evaluated two LO inhibitors MK 886 and MK 591 that are thought to act by blocking FLAP, thus preventing translocation of 5-LO from the cellular cytoplasm to the nuclear membrane [Ford-Hutchinson, 1994 #507]. We observed reproducible growth inhibition compared to vehicle control with both the quinolin-indol inhibitor MK-591 and the chlorobenzyl-butylthioindol inhibitor MK 886 (Table 2). In addition, feedback experiments with exogenous 5-HETE, demonstrated that the growth inhibition could be partially reversed, consistent with specificity of the compounds (data not shown). To examine if the cyclooxygenase pathway of AA metabolism can be inhibited in this tumor system, we tested several breast tumor cell lines in the presence of ASA. Breast tumor cell lines were not significantly growth inhibited even by the addition of high (100 uM) exogenous doses of ASA (Table 2). Representative data for NDGA, AA861 and MK886 on three cell lines are shown in Figure 6. The effects of the lipoxygenase inhibitors did not appear to depend on the ER-

status of the cell lines.

Evidence of apoptosis *in vitro* and *in vivo*

To test the hypothesis that an apoptotic pathway was involved in response to the LO-inhibitors, we examined four different cell lines *in vitro* after exposure to 2 uM NDGA, and MK 591. The percentage of apoptotic cells was determined as outlined in methods, and the results are summarized in Fig. 7, A. An increase in the percentage of apoptotic cells was observed in the cell lines tested with both inhibitors. The morphology of the cells in one such cell line after exposure to the two inhibitors is shown in Figure 7, B-D. As it can be observed, there is normal appearing morphology in the nuclei of untreated cells (B), compared to typical nuclear condensation of chromatin, evident in the cells exposed to both NDGA (C) and MK 591 (D). Similar morphologic changes were observed in the positive controls, including rodent testis, or normal rodent post lactating mammary gland (data not shown). To examine the role of LO inhibitors on the expression of the *Bcl-2* gene family, we performed a preliminary study, using MCF-7 cells. These cells have been reported to express high levels of *Bcl-2* [Monaghan, 1992 #525]. Using IHC analysis, we observed down regulation of *bcl-2* expression from 40% in untreated cells to <10% in MCF-7 cells treated with NDGA and MK 591. In addition, *bax* expression was induced (Fig.----). These findings correlated with the previously observed growth inhibition.

To further explore the role of the LO inhibitors as inducers of apoptosis, we utilized an *in vivo* model system. Athymic nu/nu mice

bearing heterotransplants of the human breast cancer cell line MCF-7, were administered NDGA, MK 591, or placebo. The tumor xenografts were harvested, and examined for the presence of apoptosis-induced oligonucleosomes. Figure 8 shows that in the groups of mice treated with LO inhibitor NDGA and the FLAP inhibitor MK 591, there was a significant number of cells with apoptotic nuclei as compared to the placebo group, which had low levels of fragmented DNA ($p<0.01$). This finding correlated with a statistically significant reduction in tumor size in the group of mice given NDGA as shown in Table 4. The flank tumor size was an average of 765 mm^3 in this group of mice, as compared to 2394 mm^3 for the placebo group ($p<0.02$). In the group of mice given MK591, there was not a statistically significant difference in tumor volume as compared to placebo.

Discussion

In the present study, we report that the mRNA for the 5-LO and FLAP transcripts is expressed in all the breast tumor cell lines evaluated. Conversely, 3/6 breast cancer cell lines tested, express mRNA generated by 12-LO and 5/6 express mRNA for 15-LO. In addition, we have identified the expression of 5-LO mRNA in malignant breast tumor tissue. Of interest, is our observation that strong expression of 5-LO could be found in morphologically normal breast tissue. Two exogenously added growth factors, IGF-I and TF, increased the endogenous production of 5-HETE and its derivatives, suggesting biosynthesis of a major 5-LO product in the breast tumor cell lines. Metabolites generated from the 5-LO pathway, are thought to act as second messengers involved in stimulation of cell growth and differentiation [Anderson, 1992 #498]. Indeed, our previous work in lung cancer, indicated that 5-HETE is a major early metabolite produced after growth-factor stimulation, and that 5-HETE has a proliferative effect on lung cancer cell lines [Avis, 1996 #428]. We now show that the 5-LO metabolite 5-HETE has a tumor proliferative effect when added exogenously to breast cancer cells *in vitro*. These results taken collectively, suggest that the 5-LO pathway of fatty acid metabolism may be the more important AA pathway for regulating breast cancer growth. This finding is in contrast to recently published work using W256 rat Walker carcinosarcoma of hematopoietic origin, which suggests that, for that tumor system, the 12-LO pathway is important in regulating growth

[Tang, 1996 #503] Other published data suggest that some tumors may produce endogenous 12(S)-HETE, that may play a role in tumor cell metastasis [Liu, 1994 #508; Chen, 1994 #398]. These data taken together with our findings in breast and lung cancer, emphasizes the complexity of eicosanoid biosynthesis in cancer. These results furthermore suggest that different tumors may express unique sets of enzymes, hence a clear understanding of these metabolic pathways will be important when designing specific experimental or therapeutic studies.

It has been proposed that inhibitors of LO reactions can act through a number of mechanisms, including redox mechanisms [Ford-Hutchinson, 1994 #507]. Our results demonstrate that a natural LO inhibitor, NDGA, which is a free radical inhibitor of lipoxygenase activity via a redox mechanism, was a potent inhibitor of growth in our study. NDGA is known to be an antioxidant, and has previously been shown to suppress DNA synthesis in the transformed human monocyte/macrophage cell line U937[Ondrey, 1989 #333]. NDGA also inhibits 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion in a mouse skin carcinogenesis model [Yamamoto, 1992 #334]. A more specific 5-LO inhibitor, the benzoquine AA861, which is notable for its structural similarity with natural quinone, also inhibited some breast cancer cell line growth *in vitro*. Two FLAP inhibitors MK 886 and MK-591, previously having been shown to inhibit leukotriene biosynthesis *in vivo* [Tagari, 1993 #426], were the most effective inhibitors of growth *in vitro* in our study. These inhibitors are thought to bind to a binding site on FLAP, indirectly preventing an association of 5-LO with FLAP [Ford-Hutchinson, 1994 #507]. Recent data

reports that there is structural similarity between FLAP and the leukotriene LTC_4 synthase, and that MK 886 inhibits LTC_4 synthase [Lam, 1994 #512]. Of the inhibitors used in our study, AA861 was the weakest inhibitor. It is possible that many complex interactions may be occurring between the competing lipoxygenase pathways, and shunting may account for the difference of the activity of the inhibitors, as recently suggested [Hussey, 1996 #509].

Our results show that COX-1, and COX-2 transcripts (6/6, and 4/6 respectively) were expressed in the cell lines. However, the cyclooxygenase inhibitor ASA, was not an effective inhibitor even at high concentrations, as shown in Table 2. A recent study using a chemically induced rat mammary carcinogenesis model system, reported that the apparent inhibitory effect of sulfone metabolite on *in vivo* carcinogenesis was independent of the cyclooxygenase pathway [Thompson, 1997 #513]. This finding is in agreement with other published data, suggesting that there is little correlation with inhibition of cyclooxygenase activity and tumor cell reduction in breast cancer [Fulton, 1984 #490; Feldman, 1985 #491]. The 5-LO product leokotriene B_4 has been reported to be involved in mammary tumor promotion, suggesting that the inhibition of tumorigenesis in that study may be due to inhibition of the LO pathway, rather than the cyclooxygenase pathway [Abou-El-Ela, 1989 #499].

Although the exact mechanism by which 5-LO inhibitors exert inhibitory effects on breast cancer cells is not clear, our results as demonstrated both *in vitro* and *in vivo*, suggest stimulation of or re-establishment of apoptotic growth regulation, resulting from the

interruption of the 5-LO pathway, as a possible mechanism for the anti-proliferative effect of the lipoxygenase inhibitors. Induction of apoptosis have been associated with several different second messenger systems [Williams, 1993 #446]. Damage-related inducers of apoptosis include anti-metabolites, oncogenes, free radicals, and oxidized intermediates of lipids and other proteins. Apoptotic cell death is characterized morphologically by cell shrinkage, membrane blebbing, cytoplasmic and nuclear condensation, and chromatin fragmentation, resulting in elimination of the dying cell without induction of an inflammatory response [Wyllie, 1980 #505]. Our *in vitro* studies show typical apoptotic morphology in the breast cancer cell lines treated with inhibitors. Modulators of apoptosis include members of the *bcl-2* gene family, which has been shown to supress apoptosis in a variety of cell types [Jacobson, 1993 #524; Williams, 1993 #446]. *BCL-2* is over expressed in 70% of breast cancers, and has been postulated to play a role in breast cancer development [Sumantran, 1995 #471]. Our observation using IHC analysis, that *bcl-2* expression is down-regulated, and *bax* expression is initiated after treatment with two LO inhibitors, correlated with a reduction of tumor growth *in vitro*. In the *in vivo* experiments, NDGA was more effective than MK 591 as an inducer of apoptosis, and this effect correlated with a reduction in tumor size. There was no side effects observed with NDGA in this group of mice, whereas some local toxicity occurred at the injection site with MK 591. Although this toxicity was reversible when the dosage of MK 591 was reduced, no statistically significant cytoreduction was observed. Because of the chemical nature of the drugs, the route of drug administration was

different between the two groups, NDGA was administered in the drinking water, and MK 591 was administered s.c. via injection.

Our result suggests that IGF-R mediated growth stimulation can be neutralized by blocking the 5-LO pathway of AA metabolism in breast cancer cell lines. These findings are consistent with Baserga's hypothesis regarding the critical role the IGF-R signaling circuit (and other growth factors/oncogenes) play in permitting cells to cycle through apoptotic growth control, possibly by altering common signaling components [Baserga, 1994 #441]. Our previously published data, using cell lines derived from an advanced stage of lung carcinogenesis, suggest that the responsiveness to apoptotic growth control was conserved late into the natural history of a cancer cell [Avis, 1996 #428]. This finding is also consistent with other published data [Piazza, 1995 #437; Tang, 1996 #503; Thompson, 1997 #513] suggesting the induction of apoptosis as a potential mechanism of growth inhibition, results which warrant further investigation.

Synthesized products of phospholipids such as AA and lysophospholipids, are potent mediators of cellular proliferation via signaling through membrane receptors. The experiments described in this paper, show a direct correlation between AA inhibition (modulated by changes in lipoxygenase synthesis, and/or enzyme inhibition), initiation of an apoptotic pathway, and tumor growth. Modulation of AA signal transduction pathways by specific inhibitors may provide a new tool to inhibit the growth of existing tumors, and to prevent the ongoing

multistage process of carcinogenesis in epithelial tumor systems. The effective drug concentrations needed to inhibit cancer cell line growth with the compounds used in our study are achievable in humans [Tagari, 1993 #426]. In contrast to the usual toxicity of chemotherapeutic agents, the modest clinical toxicity of the downstream AA inhibitors make them appropriate for consideration not only for cancer treatment, but also for chemopreventative applications.

Table 1. mRNA Expression in Breast Cancer Cell Lines

Cell line	5-LO	FLAP	LTC4	12-LO	15-LO	COX-1	COX-2
ER-Positive							
MCF7 (WT)	+	+	+	+	+	+	-
ZR75	+	+	+	+	+	+	-
T47D	+	+	+	-	+	+	+
ER-Negative							
SKBR3	+	+	+	-	-	+	+
MB231	+	+	+	-	+	+	+
MCF7-adr	+	+	+	+	+	+	+

mRNA expression of *5-LO, FLAP, LTC4, 12-LO, 15-LO, COX-1* , *COX-2* , as analyzed by RT-PCR and southern blot analysis in six different breast cancer cell lines. The experiment was repeated twice.

Table 2: Effect of Exogenous 5-HETE on Growth Proliferation

Cell Lines	uM 5-HETE	% Maximum Growth
ER-Positive		
MCF-7 (WT)	0.1	50
ZR-75	0.1	40
T47D	0.01	25
ER-Negative		
SKBR3	0.1	<10*
MB231	0.02	30
MCF-7 (ADR)	ND	

Percent maximum growth was calculated from the optical density value from a minimum of 6 replicates from at least three different experiments per cell line. Percent growth inhibition compared to vehicle control was significantly different ($p<0.05$) except where specified. (* indicates no significant difference in growth compared to control). ND: not done

Table 3. Growth Inhibition in Breast Cancer Cell Lines in the Presence of Specific Inhibitors of the AA Metabolic Pathways

Cell Lines	NDGA	AA861	MK886	MK591	ASA
ER-Positive					
MCF7 (WT)	74 ± 13	58 ±12	88 ± 7	100 ± 0	<10*
ZR-75	57 ± 28	33 ± 6	92 ± 10	95 ± 5	<10*
T47D	83 ± 6	<10*	53 ± 6	60 ± 0	<10*
ER-Negative					
SKBR3	73 ± 12	<10*	77 ± 15	90 ± 0	<10*
MB231	65 ± 21	<10*	90 ± 17	93 ± 11	<10*
MCF7-adr	75 ± 9	60 ± 23	72 ± 19	100 ± 0	<10*

The mean growth inhibition ± s.d. of a minimum of 3 experiments is presented, and drug concentrations used were 5 uM for NDGA, MK 886, and MK591, 10 uM for AA861, and 100 uM for ASA. For each compound the drug concentration that showed the greatest mean inhibition is shown. All values were determined by assessment of % growth inhibition calculated from the optical density value, with a minimum of 6 replicates from at least three different experiments per cell line. Percent growth inhibition compared to vehicle control was significantly different ($p<0.05$) except where specified. (* indicates no significant difference in growth compared to control).

Table 4. Effect of LO Inhibitors on MCF-7 Xenografts

Compound	Tumor volume mm^3
PBS	2394 ± 591
MK591	1318 ± 342 ($p>0.05$; NS)
NDGA	765 ± 142 ($p<0.02$)

Nude mice were injected with MCF-7 (WT) cells, and xenografts formed after one week. The mice were administered daily MK 591, NDGA or PBS for weeks 2-4, and the tumors were measured biweekly. The mean value ± S.E. of 5 determinations is indicated.

Figure legends:

Figure 1: Schematic outline of AA metabolism. The released arachidonic acid is metabolized either by cyclooxygenase pathway (right), or the lipoxygenase pathway (left). The proposed site of action for the inhibitors used in the study is shown in parenthesis.

Figure 2. Southern blot analysis showing mRNA expression of 5-LO and its associated activating protein (FLAP) in 6 different breast cancer cell lines (MB-231, MCF-7 WT, MCF-7 ADR, ZR-75, T47D, and SKBR-3) using RT-PCR.

Figure 3: Detection of 5-LO mRNA by in situ RT-PCR in paraffin sections taken from morphologically normal breast tissue (A, B), and malignant breast tumor tissue (C, D). Negative controls (B, D) were performed by substituting the primers by water in the PCR mixture. Magnification: A, B x67; C, D x333.

Figure 4. Production of endogenous 5-HETE after exposure to two growth factors. The cell lines used were MCF-7 WT; ZR-75; T47 D; and SKBR3. The result is presented as pMOL/0.1 ml 5-HETE and its derivatives released after exposure to 10 ug/ml TF (B), and 5ug/ml IGF-I (A), using a RIA kit. A: Baseline values. Error bars indicate SEM, and all 5-HETE values were statistically significant from baseline values ($p<0.05$).

Figure 5. Effect of exogenous addition of 5-HETE on the proliferation of breast cancer cell line ZR-75, in the presence of medium containing insulin and selenium. A representative experiment is shown, and the result is expressed as mean optical density ± standard deviation of a six data points. Asterisks indicates significant stimulation as compared to vehicle control ($p<0.05$). Maximal stimulation was observed at 0.05 mg/ml 5-HETE.

Figure 6. Effect of inhibitors of AA metabolism on breast cancer cell lines. Representative experiments on breast cancer cell lines ZR-75 (A), MCF-7 WT (B), and SKBR-3 (C) is shown for each compound. The inhibitors are NDGA (open squares), AA861(closed squares) and MK886 (closed circles). The experimental conditions were as outlined in Methods, and the results are expressed as % growth ± standard deviation, with no drug added serving as control and expressed as 100% growth. Asterisks indicates significant growth inhibition versus control ($p<0.05$). Dose concentrations used were 0.5, 1, 5 and 10 mM with all three compounds.

Figure 7: Effect of NDGA on the in vitro proliferation after exposure to IGF-I.

Figure 8: *In vitro* apoptosis analysis after treatment with two inhibitors. Results are presented as percent apoptotic cells (A) in four breast cancer cell lines (MB-231, SKBR-3, T47 D, and ZR-75). Control (filled bars),

treatment with 2 uM MK 591 (shadowed bars), and treatment with 2 uM NDGA (open bars). Morphological changes in breast cancer cell line MB-231 treated with 2 uM NDGA (C), or 2 uM MK 591 (D) as compared to control (B). Magnification: x 290.

Figure 9: Effect of administration of two inhibitors on apoptosis *in vivo*. Histological sections were stained for apoptosis in heterotransplanted tumors of breast cancer cell line MCF-7 (WT). Data represent the average ± standard deviation in ten microscopic fields counted. The difference between the treated samples and control samples was statistically significant ($p<0.05$).

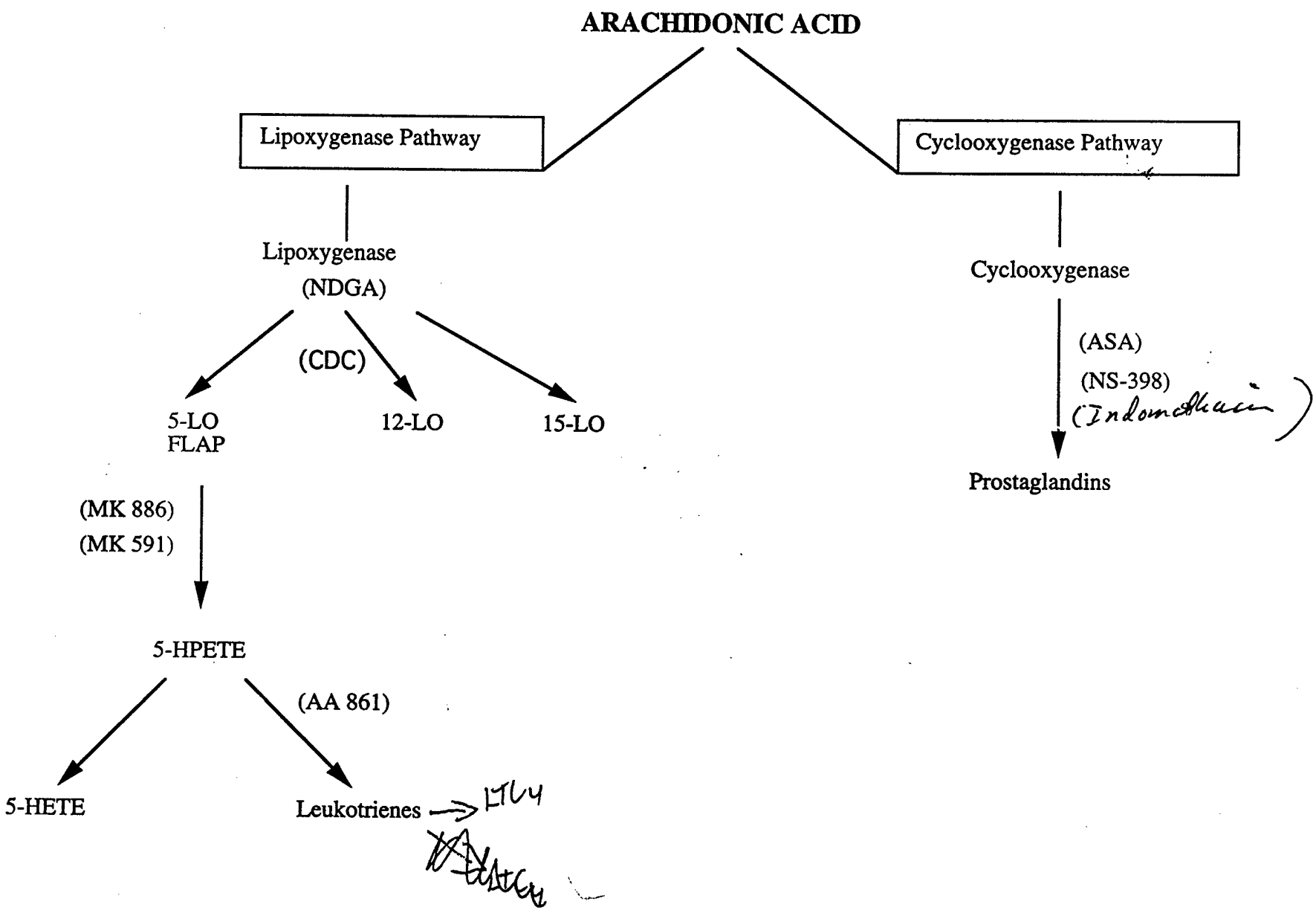
Acknowledgment:

We thank Dr. G. Allred for the kind gift of the pathological breast specimens, and Dr. B. Murphy for helpful discussions. We also thank Merck Frosst Centre for Therapeutic Research (Pointe Claire-Dorval, Quebec, Canada), for the kind gift of FLAP inhibitor MK-591. In addition, we are grateful to Amy Guzzone, and Valerie McKey for excellent technical assistance. Partial support for this work was provided by the Leila Y. and Harold G. Mathers Charitable Trust.

Abbreviations used in this paper:

AA, arachidonic acid; LO, lipoxygenase; COX, cyclooxygenase; FLAP, 5-lipoxygenase activating protein; IGF-R, insulin-like growth factor receptor; ER, estrogen receptor; RT-PCR, reverse transcriptase polymerase chain reaction; RIA, radioimmunoassay; ASA, acetylsalicylic acid; NDGA, nordihydroguaiaretic acid;

FIGURE 1


ARACHIDONIC ACID
Lipoxygenase Pathway
Cyclooxygenase Pathway
Lipoxygenase
(NDGA)
Cyclooxygenase
(CDC)
(ASA)
(NS-398)
(Indomethacin)
5-LO
FLAP
12-LO
15-LO
(MK 886)
(MK 591)
Prostaglandins
5-HPETE
(AA 861)
5-HETE
Leukotrienes
LTC4

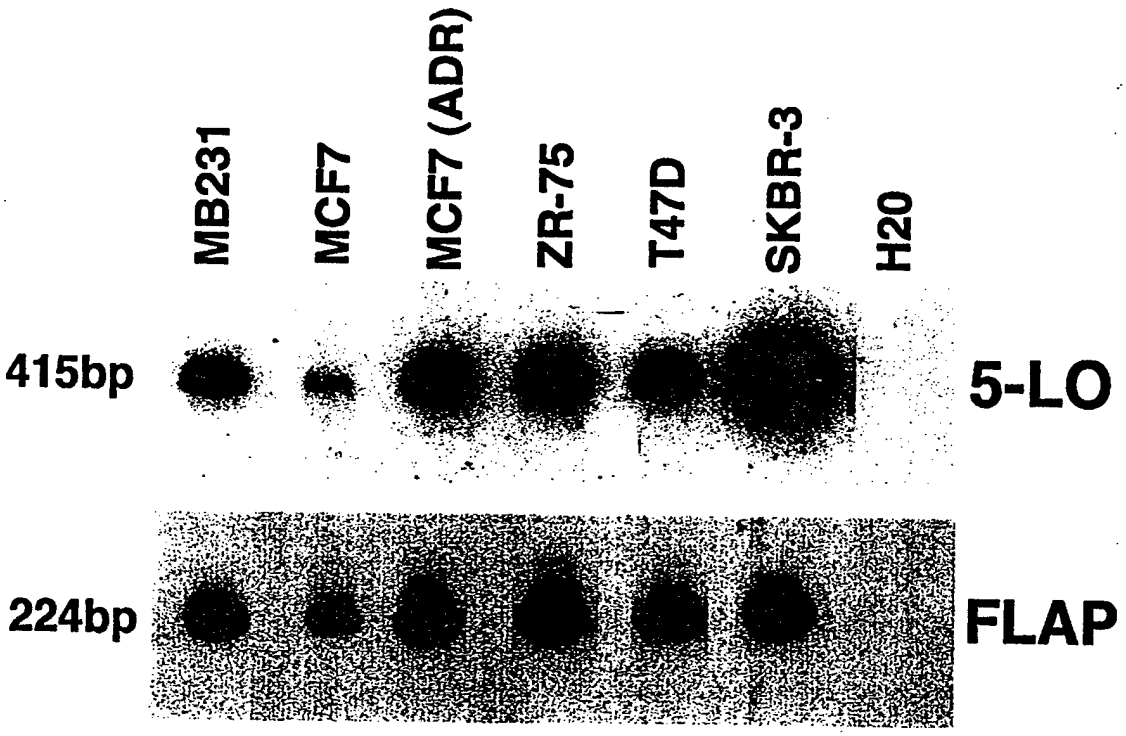


FIGURE 2

Human breast tumor

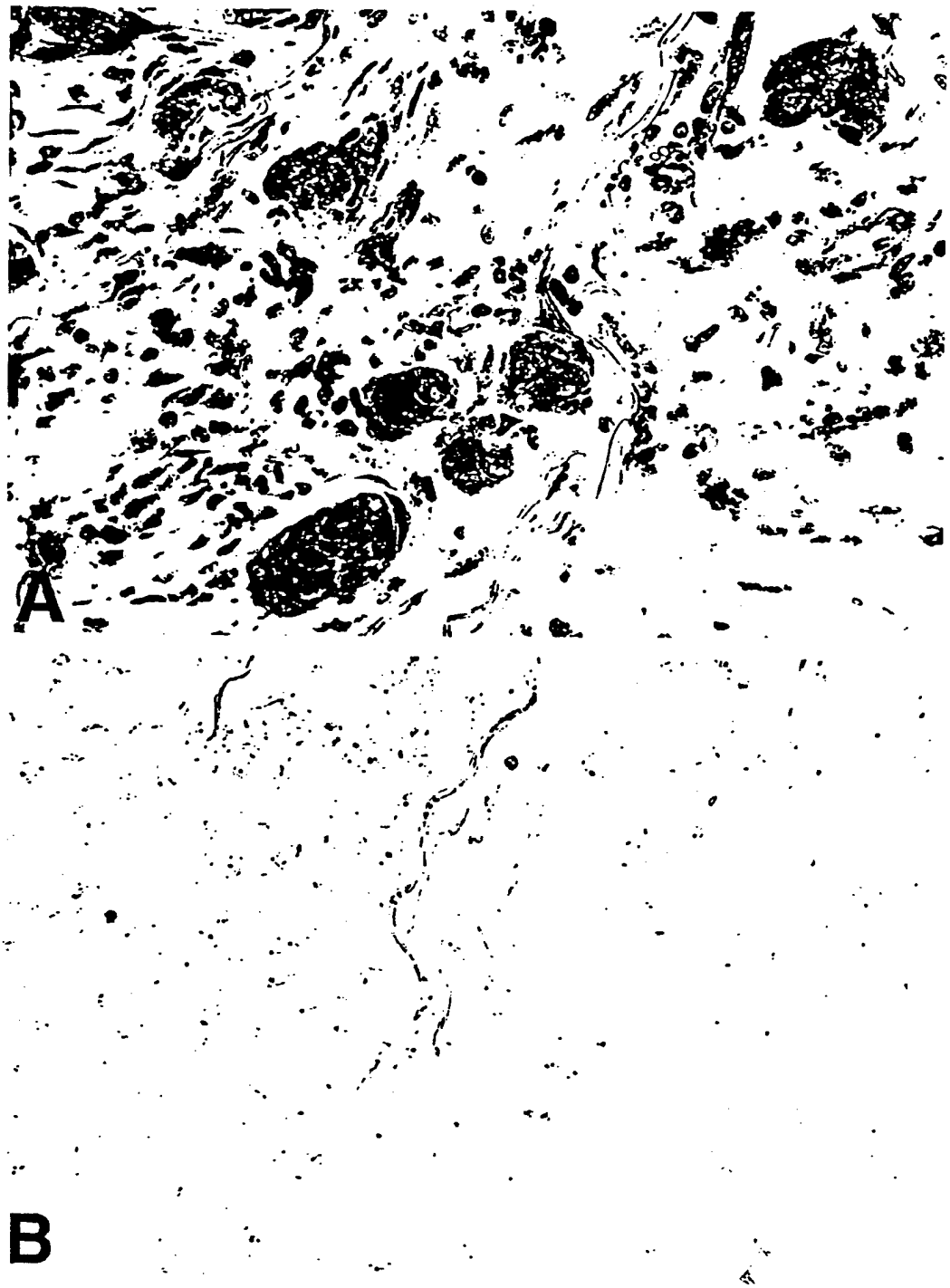
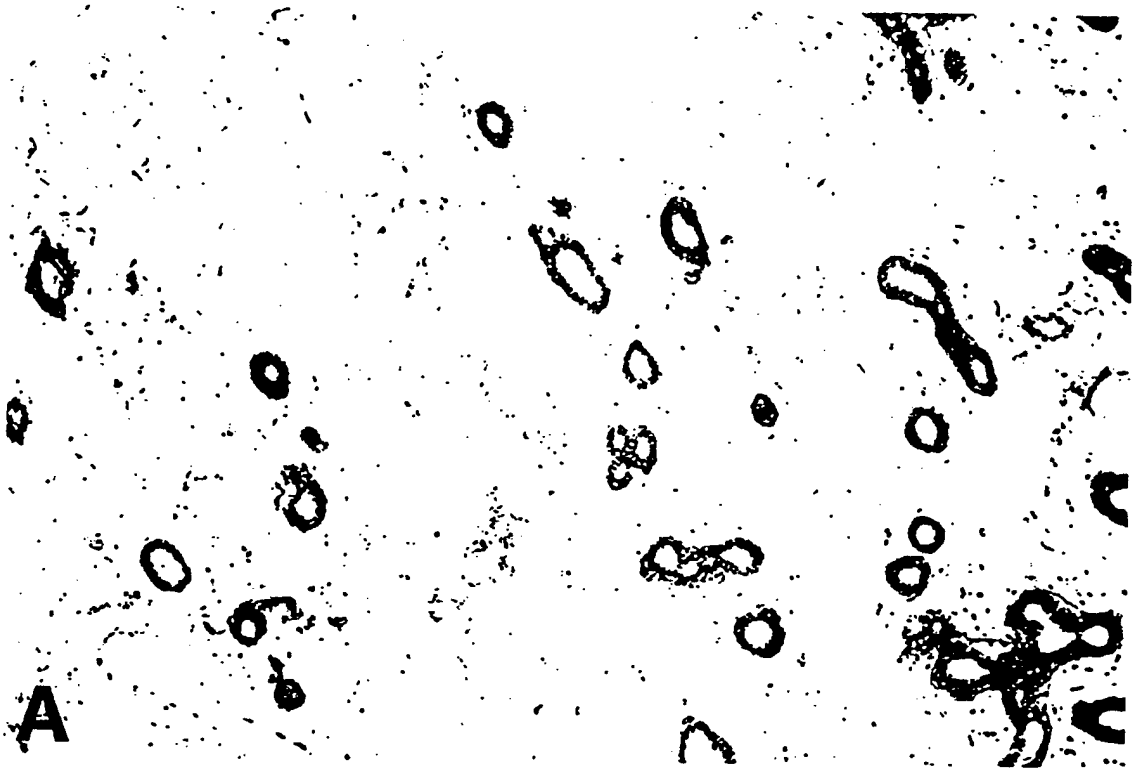


FIGURE 3A

Normal human breast tissue



B

FIGURE 3B

FIGURE 4

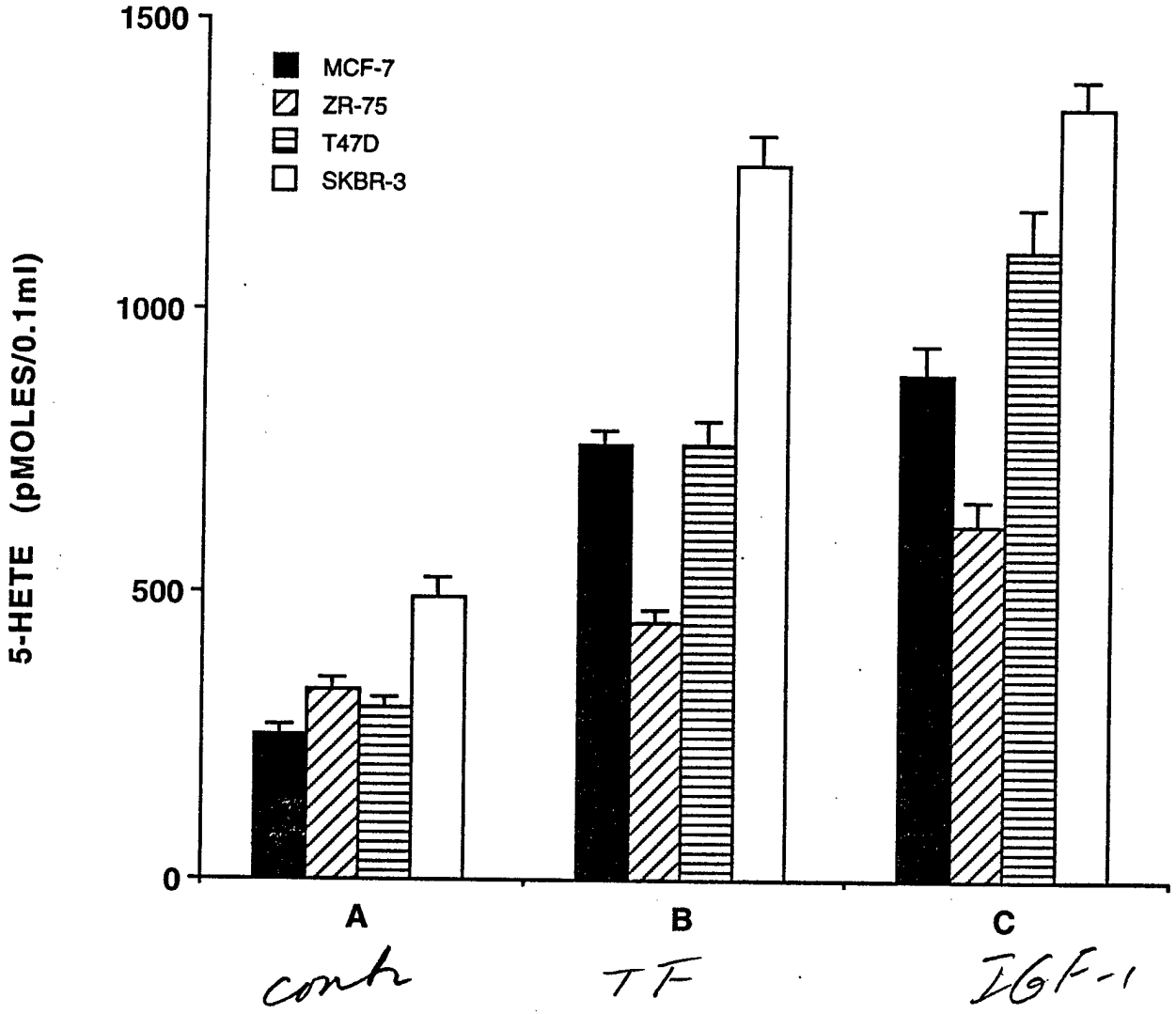

1500
1000
500
0
5-HETE (pMOLES/0.1ml)
MCF-7
ZR-75
T47D
SKBR-3
A
B
C
cont
TF
IGF-1

FIGURE 5

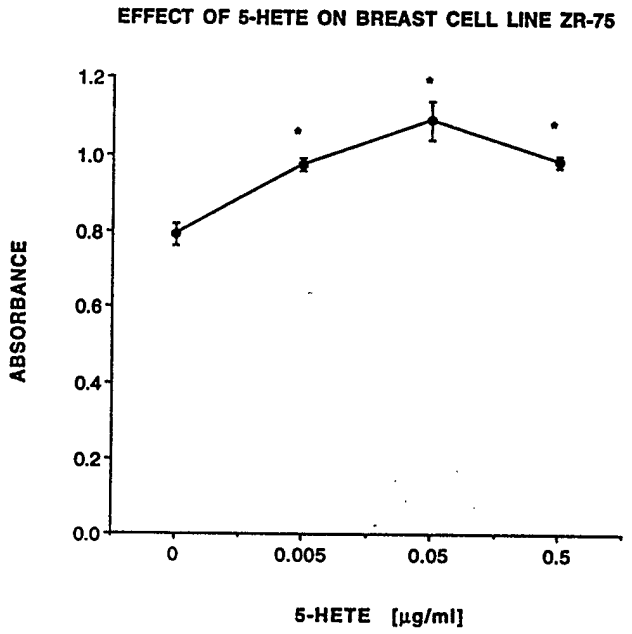

EFFECT OF 5-HETE ON BREAST CELL LINE ZR-75
ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
0.005
0.05
0.5
5-HETE [µg/ml]

FIGURE 6

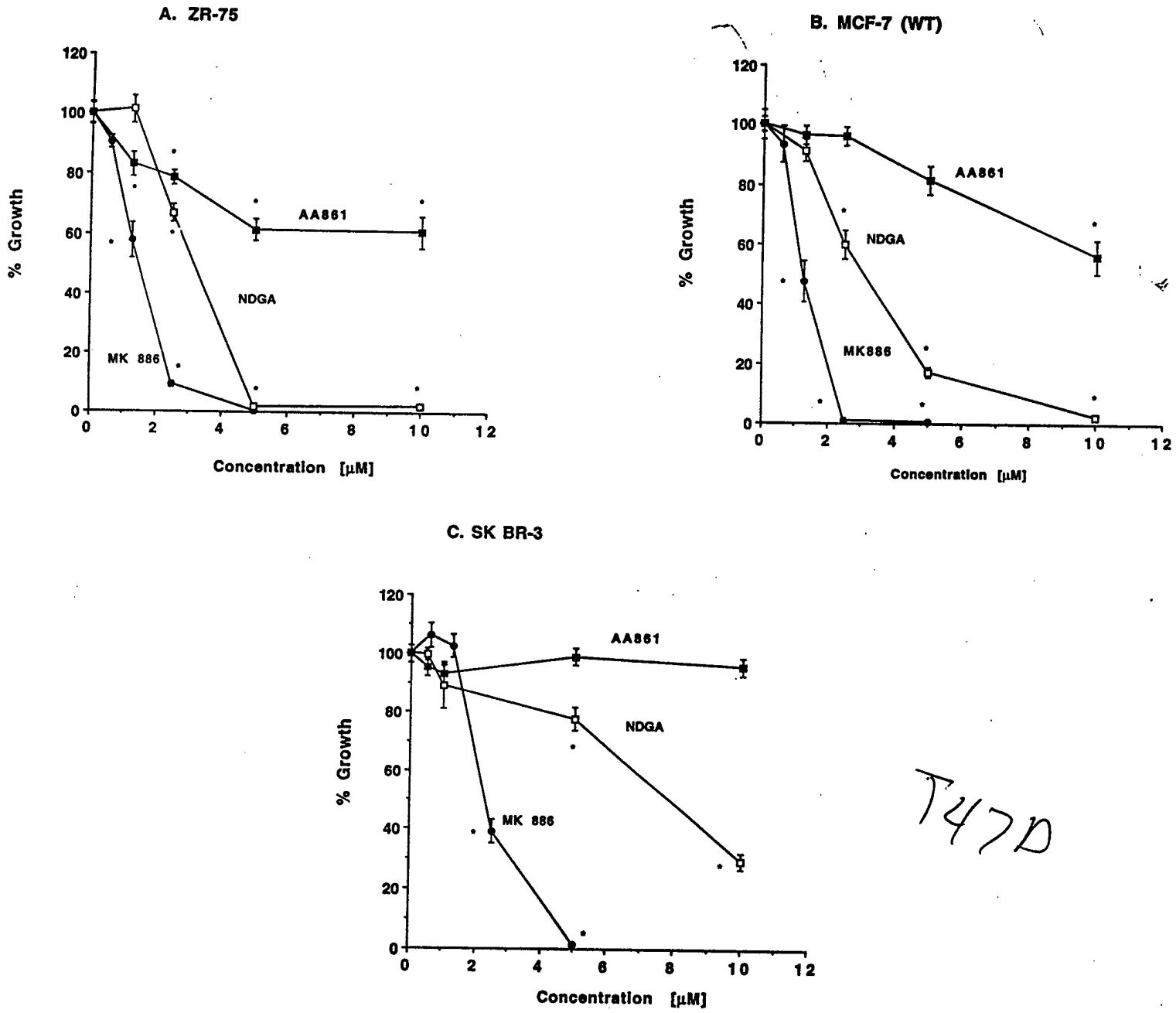

A. ZR-75
% Growth
Concentration [µM]
AA861
NDGA
MK 886
B. MCF-7 (WT)
% Growth
Concentration [µM]
AA861
NDGA
MK886
C. SK BR-3
% Growth
Concentration [µM]
AA861
NDGA
MK 886
T47D

FIGURE 7A

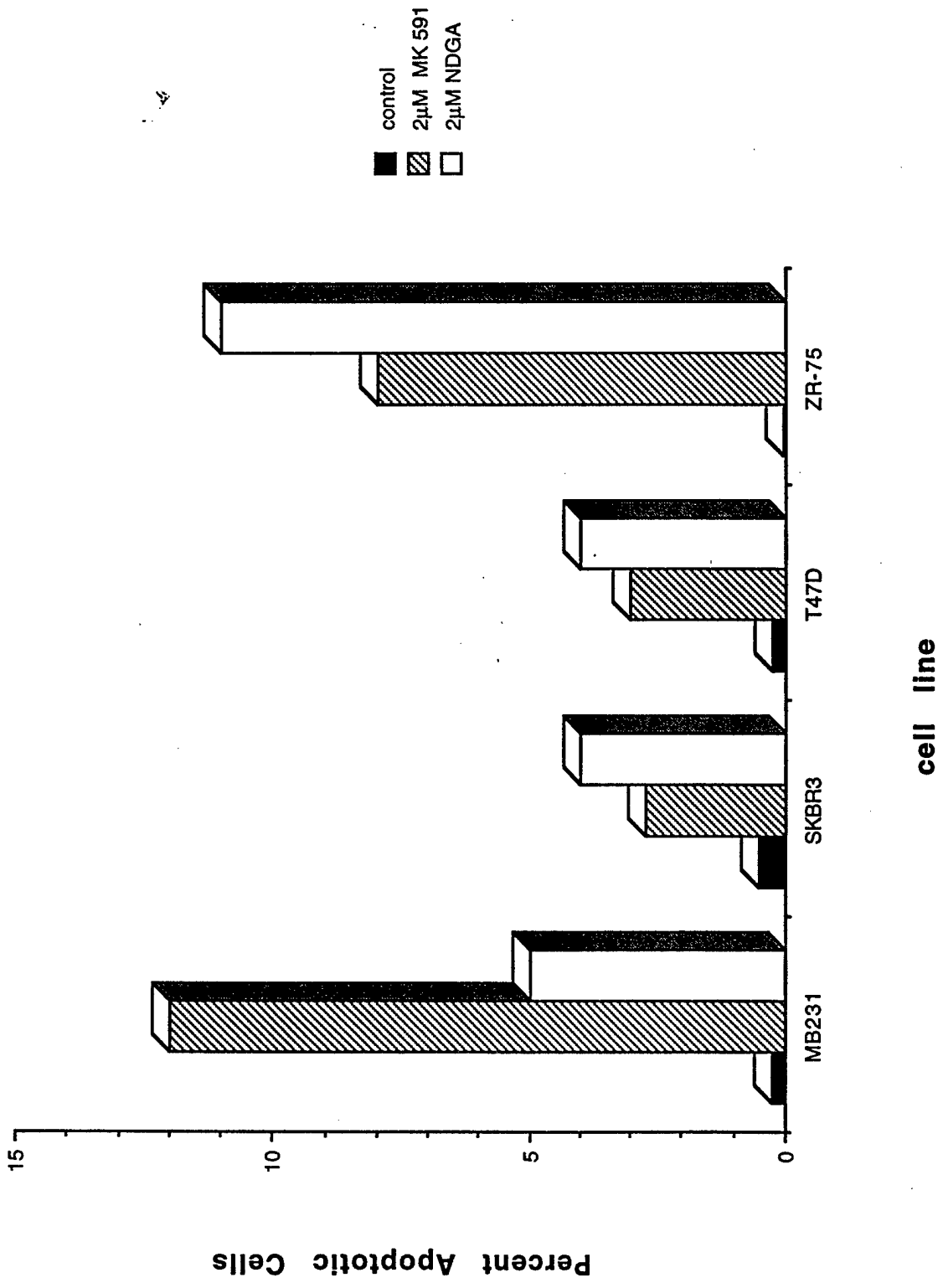

control
2μM MK 591
2μM NDGA
Percent Apoptotic Cells
0
5
10
15
MB231
SKBR3
T47D
ZR-75
cell line

FIGURE 7 CONTINUED

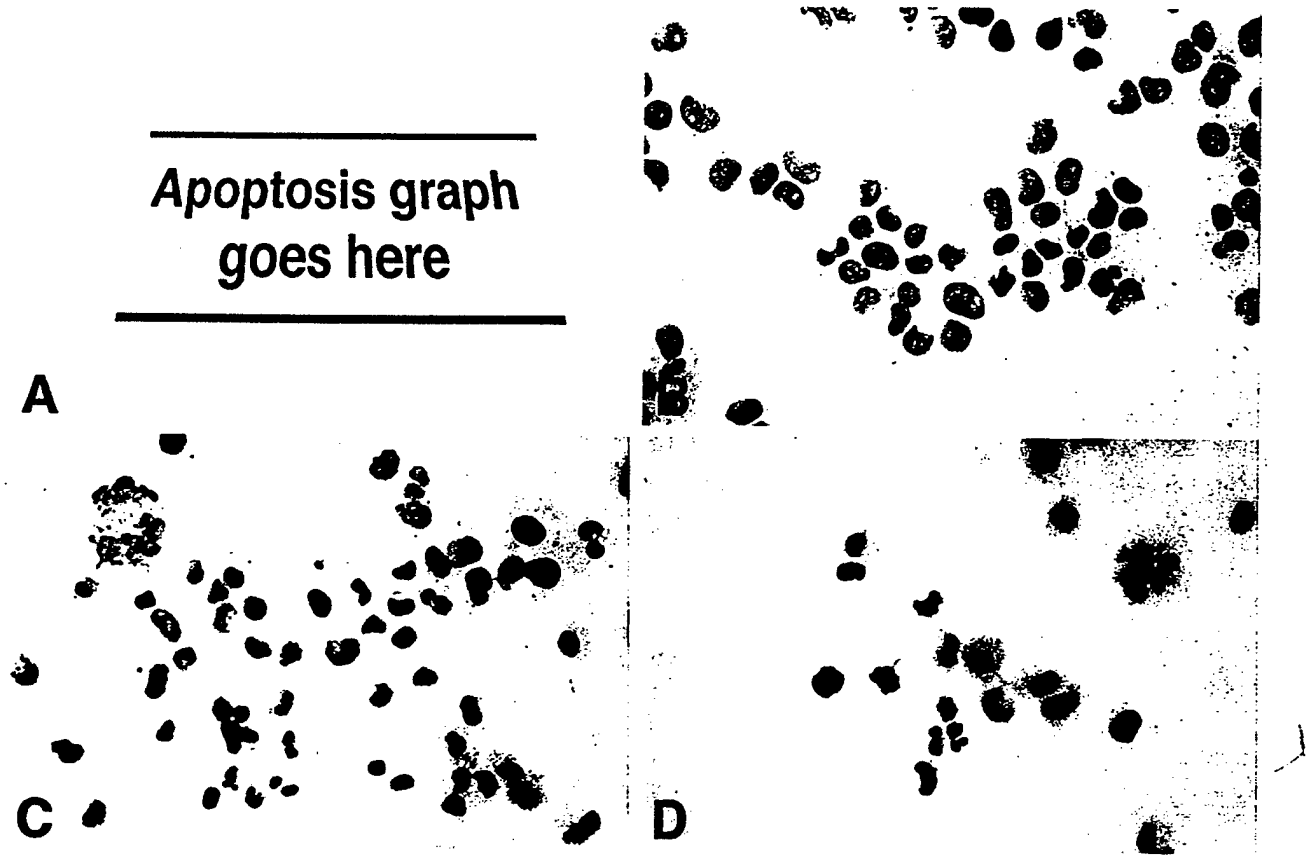
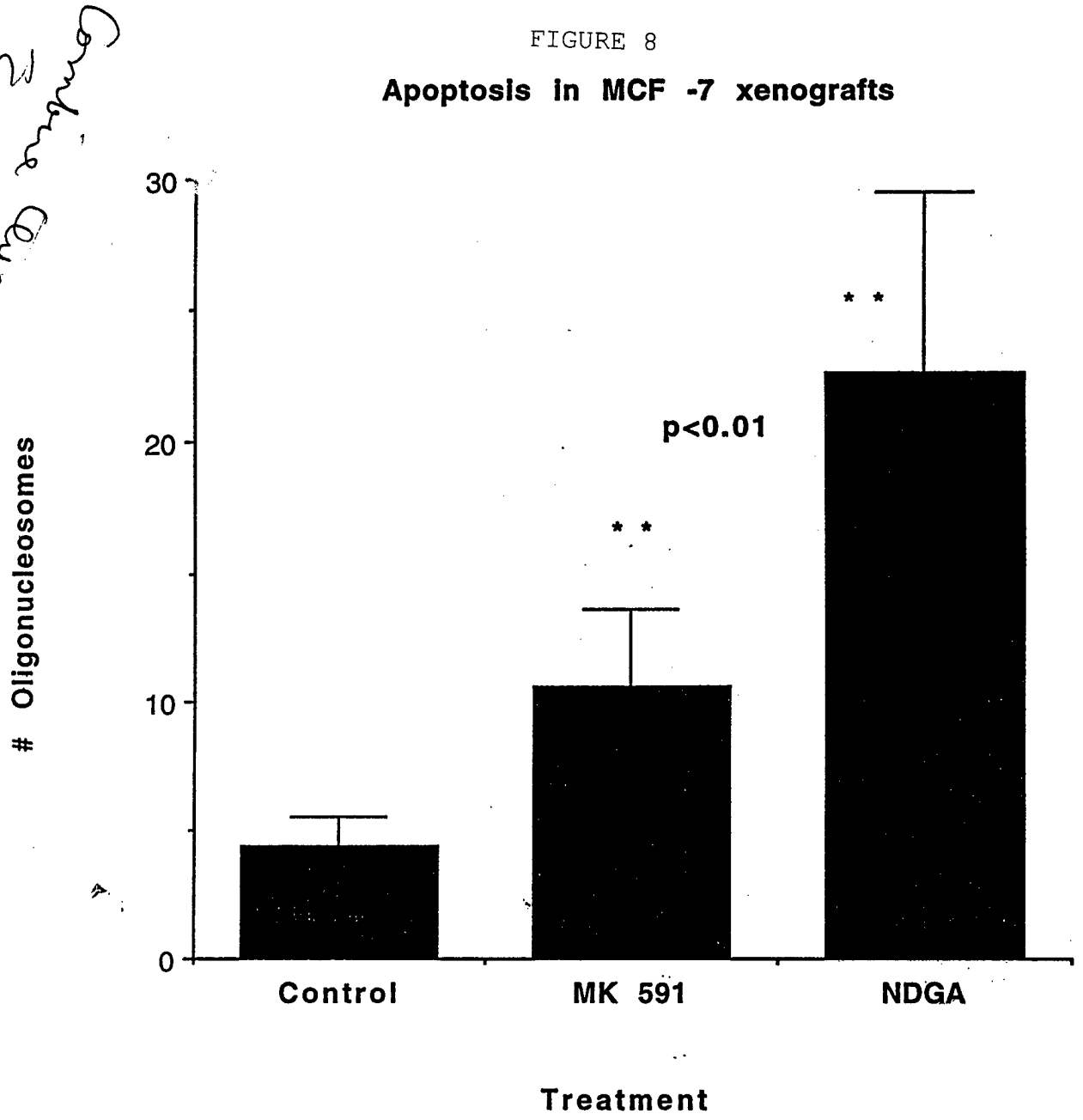


FIGURE 8


Apoptosis in MCF -7 xenografts
Oligonucleosomes
30
20
10
0
p<0.01
* *
* *
Control
MK 591
NDGA
Treatment

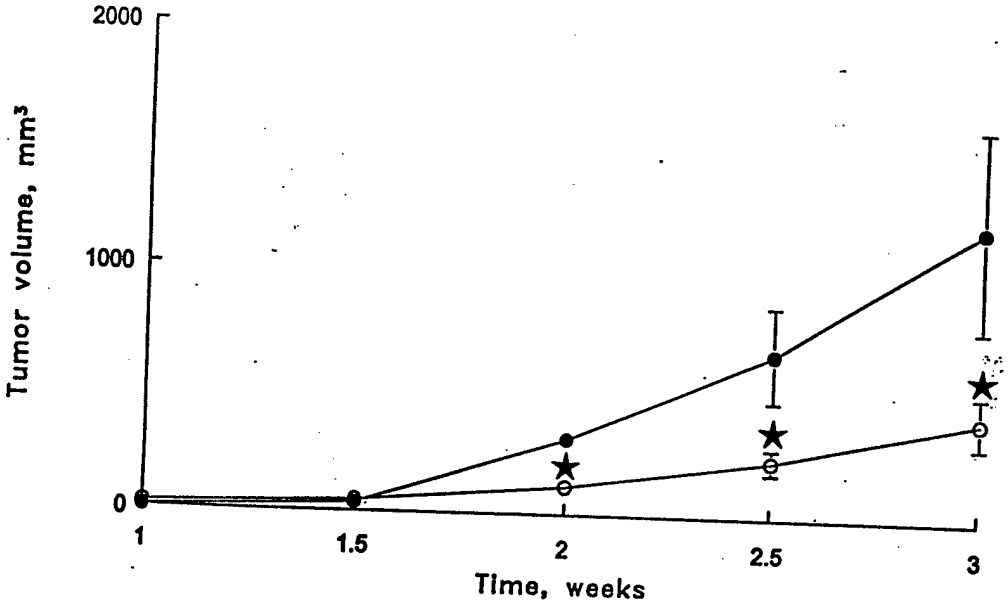


TABLE 1

Control of human breast cancer cell growth by manipulation of arachidonate metabolism.

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Key Words: 5-LO, Arachidonic Acid pathway, bioactive lipids, MCF-7, bone marrow cells, signaling

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ABSTRACT

Arachidonate metabolites are important regulators of cell growth in human breast cancer. Production of bioactive lipids is initiated by the enzyme phospholipase A2 which releases arachidonic acid (AA); AA is rapidly metabolized by cyclooxygenases (CO) or lipoxygenases (LO) to other highly potent lipids. In the present study we used inhibitors which blocked specific pathways in arachidonate metabolism. Many of these drugs proved to be equally effective in blocking proliferation in both MCF-7 wild type (WT) and MCF-7 multidrug resistant (ADR) human breast cancer cell lines and were non toxic to cultures of human bone marrow cells. Inhibitors of 5-LO pathway (MK-591, MK-886, and AA-861) were most effective in blocking proliferation of both of these breast cancer cell lines while CO inhibitors (indomethacin, aspirin, etc.) were not effective. Curcumin, an inhibitor of both cyclooxygenase and lipoxygenase pathways of eicosanoid metabolism, was equally effective in blocking proliferation of the multidrug-resistant cells, MCF-7 ADR^{10} and WT cells. Cell cycle studies showed curcumin to arrest cells at G1 leading to apoptosis. Protein kinase C (PKC), the activation of which is intertwined with arachidonate metabolism, also proved to be a good target since inhibitors of PKC effectively blocked proliferation in these cultures. PCA-4248, an inhibitor of platelet activating factor generation (a byproduct of arachidonate release), was an effective antiproliferative agent. Thapsigargin, a drug which induces the release of stored calcium, blocked proliferation in MCF-7 WT (IC50= 5 nM) but was ineffective with MCF-7 ADR^{10} cells. Inhibitors that effectively block the proliferation of breast cancer cells showed varying degrees of toxicity to cultures of human bone marrow cells. We observed greater toxicity to bone marrow cells using inhibitors that interfered with the utilization of arachidonic acid in contrast to those that blocked utilization of its downstream metabolites. MK-591, MK-886, PCA-4248, Curcumin and AA-861 blocked proliferation of breast cancer cells but showed little toxicity to bone marrow cells and may be potentially useful in human breast cancer therapy.

INTRODUCTION

Epidemiologic investigations have suggested an association of dietary fat intake with breast cancer risk. Bioactive lipids generated from these fat metabolites are known to increase proliferation in cancer cells. Various studies have suggested dietary fat content, especially polyunsaturated fatty acids, promotes tumor growth by increasing synthesis of eicosanoids, particularly AA products (Wynder et al. 1986; Welsch and Aylsworth, 1983, Carter et al. 1983). The possible role of arachidonic acid derived eicosanoids as regulators of neoplastic cell growth is an area of significant interest in breast cancer biology.

Phospholipase A2 (PLA2) is the family of enzymes which specifically hydrolyzes the 2-acyl position of glycerophospholipid. It has been reported that the concentration of PLA2 was elevated in the lungs, breasts, and the digestive organs of patients with malignant tumors and that the incidence and magnitude of the elevation increased with advanced cancer stage [4,5]. In our previous work with wild type (WT) and drug-resistant (MCF-7 ADR^{10}) MCF-7 cells, we observed PLA2 activity with specificity toward either linoleoyl or arachidonyl phosphatidylinositol [6]. PLA2's are usually most efficient with polyunsaturated fatty acids in the SN-2 position, which result in the release of arachidonic acid (AA). Activation of AA metabolism is initiated by the release of AA from the phospholipid pool by the enzyme phospholipase A2 (Axelrod et al. 1988). AA is metabolized through the cyclooxygenase pathway which results in prostaglandins production (Boyle et al. 1994) or through the 5-lipoxygenase (5-LO) pathway, which results in the production of leukotriene (Henderson, 1994). Both prostaglandins and leukotriene directly stimulate the growth of malignant cells (Lee and Ip, 1992; Snyder et al. 1989).

Metabolism of exogenous arachidonic acid by lipoxygenase or cyclooxygenase pathways produces a myriad of highly potent bioactive lipids which include leukotrienes, HPETEs, HETEs, and prostaglandins. Many of these metabolites have been shown to play a significant role in cancer cell growth. The arachidonate-derived eicosanoids PGE2, LTB4, and 5-, 12-, and 15-HETEs have been shown to be significantly higher in human

breast cancer cells than control cells [refs]. In Swiss 3T3 cells, stimulation of DNA synthesis occurs predominantly by activation of arachidonic acid release, followed by its oxidation to PGE2 and stimulation of adenylyl cyclase [refs]. Metabolites of arachidonic acid and linoleic acid served as regulators of the EGF transduction system in Syrian hamster embryo fibroblasts [refs]. Initiation of growth of human myeloblastic leukemia cells is dependent upon the increased formation of AA and its derivatives, formed primarily via the lipoxygenase pathway and the initiation of growth in these cells was followed by the rapid release of AA, HETEs and phospholipids into the culture medium [refs]. The effects of lipoxygenase and cyclooxygenase metabolism inhibitors on a human gastric cell line derived from a stomach tumor was suppression of cell proliferation [refs]. The consequent alteration in PKC, catalyzed by phospholipase(s) activity in endothelial cells, regulates the growth-dependent changes in AA release [refs].

Avis et al. reported that exogenous addition of 5-HETE was found to stimulate lung cancer growth in vitro [Avis, 1996 #16]. When selective antagonists were used to inhibit 5-lipoxygenase metabolism, significant growth reduction resulted in a number of lung cancer cell lines. Similarly, LTB4 and 12(R)-HETE significantly increased proliferation of two colon carcinoma cell lines, HT-29 and HCT-15 [Bortuzzo, 1996 #17]. However, isomers of these two compounds such as LTB5 and 12(S)-HETE failed to affect the proliferation rate of these two cell lines. This demonstrates the importance of specificity in cancer cell proliferation. Epidemiology studies show that death rates from colon cancer decreased 40% for individuals who took aspirin (AA inhibitor) more than 16 times/month [7]. The use of inhibitors to manipulate AA pathways will help us better understand the function of elevated PLA2 levels in cancer cells, which may lead to the discovery of new anti-cancer drugs.

In the present study we have examined the effect of various inhibitors of key signaling pathways on growth of breast cancer cells. We show that inhibitors of the 5-LO pathway can block growth of breast cancer cells , especially the drug resistant MCF-7 ADR line very effectively. Inhibitors of the PKC pathway were also able to block the growth of these cells. The toxicity of these inhibitors were then tested on the growth of bone marrow cells. The selection of some of these inhibitors might be an alternative

treatment regimen for the breast cancer patients especially those that develop drug resistance during their course of treament.

MATERIALS AND METHODS

Materials. MK-591 was a gift from Merck-Frost, Pointe Claire, Quebec, Canada. All other inhibitors were purchased from BIOMOL Research Laboratories, Inc.

Cells. The human breast cancer MCF-7 Wild Type (WT) and its Adriamycin-Resistant, (MCF-7 ADR[10]) cells were obtained from Kenneth Cowan (NCI, NIH Bethesda, MD). The cells were grown in Improved Modified Essential Medium (IMEM)[4] containing 8% fetal bovine serum, 50,000 units/liter penicillin and 5,000 μg/l streptomycin at 37°C with 5% CO_2.

Human bone marrow mononuclear cells were obtained from healthy volunteer donors arranged by Poietic Technologies Inc. and used for colony assays without any prior treatment. The stroma colonies were grown in IMDM-based Long Term Culture Media[5] (LTCM) containing 25% horse serum (Hyclone) with 5% CO_2 and 100% humidity at 37°C.

Methyl (H^3)-thymidine incorporation. Inhibition of proliferation in MCF-7 WT or MCF-7 ADR[10] cultures was performed in 96-well plates. Cells were plated 15,000 cells/well in 0.2 mL IMEM culture fluid[1] and incubated overnight. Inhibitors were added (50 μl) to achieve the indicated concentration and incubated for three days. During the last 18 hours, Methyl(H^3)-thymidine (1μCi / well) was added. The cells were trypsinized and harvested using Packard Unifilter System. The filter plates were dried in the air. Then, 40μL of Packard Microscint 0 scintillation cocktail was added to each well and the filter plates were counted using Packard Top-count.

Colony Assay. Human bone marrow stroma colony studies were performed using human bone marrow mononuclear cells[2]. Cells ($2x10^5$ cells/well) were plated in 4-well plates with 5 ml LTCM and inhibitors were added on the second day. The media was changed with fresh media either with (continuous treatment) or without drugs (pulse) every week. The stroma colonies were stained on the second and fourth week of

treatment with HEMA 3 (differential hematology stain, CMS). Colonies with diameters larger than 1 mm were counted and the size of the each colony was measured.

The hematopoietic progenitor colony assay was performed using Methylcellulose-based Colony Cocktail from Stem Cell Technologies, Inc. (HCC-4434) containing 30% Fetal Bovine Serum; 50 ng/ml rh Stem Cell Factor; 10 ng/ml rh GM-CSF; 10 ng/ml rh IL-3; and 3 units/ml rh Erythropoietin. Inhibitors were premixed with the cocktail (4.5 ml) and were added with the cells and plated in a 35mm diameter gridded tissue culture plate (Nunc. Inc.). After a two week incubation period, the hematopoietic colonies were counted using an inverted phase microscope with 40X magnification. Using the standard criteria developed by Stem Cell Technologies Inc. (Atlas of Human Hematopoietic Colonies), the colonies were classified into categories that included: CFU-GM, BFU-E , CFU-E, CFU-Mix, and CFU-GEMM.

Both stroma colony and hematopoietic colony assay experiments were performed with four replicates and repeated at least three times with different lots of cells. Regardless of the lot of the cells, the same trends were observed.

RESULTS

Effect of Lipoxygenase/Cyclooxygenase inhibitors on breast cancer cell growth: Cells were plated in 96 well plates in the presence of various inhibitors of the arachidonic acid pathway and their growth was measured by 3H Thymidine incorporation. Inhibition of proliferation in MCF-7 WT or MCF-7 ADR[10] cultures with lipoxygenase inhibitors is shown in figure 1. Inhibitors of lipoxygenase (LO) pathways were most effective at blocking proliferation. The effect of MK591, MK886 and NDGA was compared between the MCF-WT (Fig 1 A) and MCF-7 ADR cells (Fig 1 B). There was a concentration dependent decrease in growth of these cells in the presence of these inhibitors. Curcumin, AA861, and ketoconazole also blocked cell growth of these breast cancer cells (Fig 1 C, D). Curcumin, a dual inhibitor of 5-lipoxygenase and cyclooxygenase, was much more effective (ca. 12 fold) at blocking proliferation of the multidrug-resistant cells, MCF-7 ADR[10] compared to MCF-7 wild type (WT) cells.

Curcumin, an agent with anti-inflammatory and anti-oxidant properties [Xu, 1997 #8]. showed maximum inhibition of growth of breast cancer cells at very low concentrations.

However, cyclooxygenase (CO) inhibitors were not effective in blocking the growth of these breast cancer cells (Table 1). Since metabolites of 5-LO has properties of a co-growth factor, it is possible that decreases in 5-LO products inhibit breast cancer cell proliferation. It is also possible that decreases in the ratio of lipoxygenase to cyclooxygenase products are responsible for the inhibition.

Effect of PKC Inhibitors on growth of breast cancer cells:

Protein Kinase C (PKC) has become an increasingly important signaling kinase in cancer research because it serves as a receptor for phorbol esters, potent tumor promoters [Hannun, 1989 #11]. We found that some PKC inhibitors such as sphingosine and chelerythrine chloride were very effective at blocking proliferation of MCF-7 WT and MCF-7 ADR^{10} cells. Inhibition of proliferation in WT or MCF-7 ADR^{10} cultures with PKC inhibitors is shown in figure 2. Sphingosine disrupts sphingolipid metabolism by preventing PKC from interacting with DAG and phorbol esters which may lead to the accumulation of lysosphingolipids and the consequent inhibition of PKC [8a]. Chelerythrine chloride causes cell cycle arrest in G2 by selectively inhibiting and degrading betaII protein kinase C, which must be activated for mitotic nuclear lamina diassembly and entry into mitosis [Thompson, 1996 #10].

Other PKC inhibitors such as H7 were ineffective in this system. H7 is a non-selective inhibitor of protein kinases which inhibits PKA, PKG, MLCK, and PKC activity [Nixon, 1991 #13].

Regulation of breast cancer cell growth by PAF inhibitors:

PCA-4248 inhibits PAF (a byproduct released from arachidonate metabolism) binding to human platelet and PMNL receptors. Inhibition of proliferation in MCF-7 WT or MCF-7 ADR^{10} cultures with PCA-4248 and thapsigargin is shown in Figure 3. Blocking PAF binding results in the accumulation of arachidonate metabolism products, which may account for the inhibition of proliferation in MCF-7 WT or MCF-7 ADR^{10} cultures by PCA-4248. Thapsigargin induces the release of intracellular Ca^{2+} without hydrolysis of

inositolphospholipids and down-regulates the EGF receptor. It inhibits drug resistant MCF-7 ADR[10] cells effectively at very low concentrations. However for MCF-7 wild type cells, there was a very small degree of inhibition at the same concentration.

Human Bone Marrow progenitor colonies in the presence of inhibitors:
For any drug to be useful for chemotherapy it is important to study the toxicity of the drugs on bone marrow cells. To determine the toxicity of these inhibitors, the ability of human bone marrow cells to form colonies was studied. Human bone marrow stroma colonies with one week of treatment with the inhibitors are shown in Figure 4 (two week colonies) and Figure 5 (four week colonies). The total number of stromal colonies, the average area of each colony, and the percentage coverage of the plate did not change in the presence of MK-591, MK-886, and PCA-4248 at IC_{50}, when compared to the control values. However, the stroma colonies were wiped out even with concentrations of the inhibitors lower than IC_{50} for NDGA and sphingosine. Lipoxygenase inhibitors (MK-591 and MK-886) did not have any toxic effect on the formation of stroma colonies. PKC inhibitors stopped the colony growth at a concentration lower than IC_{50}. However, PAF Binding Inhibitor PCA-4248, did not affect the growth of stroma colonies when the concentration was at IC_{90}. When the human bone marrow cells were treated for two weeks or four weeks continuously with the inhibitors, the growth of the colonies show similar trends to those treated for one week (Figure 6 and 7).

Figure 8 shows the total number of hematopoietic colonies in the presence of these inhibitors. The distribution of these colonies categorized as BFU-E, CFU-E, CFU-GM, CFU-mix and CFU-GEMM is summarised in Table 2. Again MK-591, MK-886, and PCA-4248 showed no toxicity on the hematopoietic colonies. The relative population of the hematopoietic colonies in these treated samples were similar to the control. NDGA and Chelerythrine chloride were toxic to hematopoietic colonies only at higher concentrations. Sphingosine and curcumin were very toxic to hematopoietic colonies even at lower concentrations. Thus the MK drugs and PCA are some of the drugs that may have some use in the clinics in future becuase they are safe and effective in blocking the growth of breast cancer cells.

DISCUSSION

There is a constant need for additional therapeutic strategies to combat the disease in order to improve the quality of life and prolonge survival for women with breast cancer. Bioactive lipids play an important role in the growth and development of the normal mammary gland . Understanding the regulatory role of these lipids on the control of the epithelial cell population is extremely important. Dietary fat content, especially polyunsaturated fatty acids, promotes tumor growth by increasing synthesis of eicosanoids particularly AA products (Wynder et al. 1986; Welsch and Aylsworth, 1983, Carter et al. 1983). The possible role of arachidonic acid derived eicosanoids as regulators of neoplastic cell growth is an area of significant interest in breast cancer biology.

Signal transduction explains the molecular events by which extracellular signals elicits an intracellular response. The generation of a variety of lipid signal transduction molecules from hydrolysis of membrane phospholipids is an early response. Several types of signal transduction pathway inhibitors effectively blocked proliferation in MCF-7 WT or MCF-7 ADR^{10} cultures. Lipoxygenase (LO) inhibitors NDGA, MK591, AA-861 and MK886 were equally effective at blocking proliferation for MCF-7 WT and MCF-7 ADR^{10} cells.

Curcumin has been shown to strongly inhibit the LO pathway and weakly inhibite the CO pathway. Curcumin inhibits proliferation by blocking the action of the Thymidine Kinase enzyme which is necessary for cell cycle progression through the S-phase [Singh, 1996 #7]. In immortalized NIH 3T3 and malignant cancer cell lines, curcumin induced cell shrinkage, chromatin condensation, DNA fragmentation, and characteristics of apoptosis. In the present study it inhibits the proliferation for both MCF-7 WT and MCF-7 ADR^{10} cells. However, the IC_{50} for MCF-7 ADR^{10} cells (12μM) is much smaller than that for MCF-7 WT cells (90μM). Interestingly, curcumin effectively blocked proliferation in only MCF-7 ADR^{10} much more than in MCF-7 WT cells. However Ketoconazole was more effective with MCF-7 WT cells than MCF-7 ADR^{10} cells

The cyclooxygenase (COX) enzyme is an important enzyme because it catalyzes the initial reaction of arachidonate metabolism that leads to the formation of prostaglandins, thromboxane, and prostacyclin [Gierse, 1996 #14]. Recently, a second form of the cyclooxygenase (COX) enzyme, COX-2, has been isolated. A single amino acid difference in the active site (valine 509 to isoleucine) and a series of differences in the active site confers selectivity for COX-2. COX-2, which can be induced by cytokines and growth factors, is linked to inflammatory cell types and tissues whereas COX-1's course of action resides primarily in the stomach and kidney [Gierse, 1996 #14]. It is possible that a selective COX-2 inhibitor may eliminate the side effects associated with COX-1 while still mainitaining COX-2 inhibition and vice versa. In the present study we did not observe any significant inhibition of breast cancer cell growth in the presence of COX inhibitors.

Since PKC is such an important regulator of cell growth and differentiation, the finding that some PKC inhibitors possess antiproliferative properties can be a very exciting area of cancer research [Budworth, 1994 #18]. PKC, which occurs as a family of isoenzymes that share similar structural features, is regulated by calcium, phospholipids, and sn-1,2-diacylglycerol (DAG). Some PKC inhibitors such as Sphingosine and Chelerythrine chloride were effective at inhibiting growth of both breast cancer cell lines while others such as H7 were not. We found that H7, which many people believe to be a potent inhibitor, to be ineffective against proliferation. As we know, there are many isozymes of PKC so it is possible that only certain isomers play an important role in MCF-7 WT cell proliferation. The problem with H7 is that many of them only possess modest degrees of specificity for PKC [Budworth, 1994 #18]. Thus there is a causal relationship between inhibition of PKC and cancer cell growth arrest.

An inhibitor of PAF (PCA-4248) effectively blocked proliferation of MCF-7 WT and MCF-7 ADR[10]. Thapsigargin, a down- regulator of the EGF receptor and PI3 kinase was very effective only with MCF-7 WT cells. Inhibitors of the LO pathway or specifically the 5-LO pathway were most effective at blocking proliferation while cyclooxygenase (CO) inhibitors were not effective. Blocking both LO and CO pathways in WT cells using ETYA, phenidone or curcumin did not inhibit the proliferation. In

general, these inhibitors were more effective blockers of CO than LO pathways. We have previously observed {Avis et al, 1996} that AA metabolism in the presence of aspirin was shifted to large increases in LO products. This might have occurred in the presence of these dual inhibitors.

NDGA, MK-591, and MK-886 block arachidonic acid metabolism pathway at different stages and all effectively inhibit the proliferation of MCF-7 WT and MCF-7 ADR[10] cultures. MK-591 and MK-886 did not exhibit any toxicity on either human bone marrow stroma colonies or on human hematopoietic colonies at a concentration lower than IC_{90} . However, NDGA completely blocked stroma colony formation even though the hematopoietic colonies still survived at a concentration lower than IC_{70}. This could be due to the fact that it blocks the initial conversion from AA to any/all of the lipoxygenases and their products. PCA-4248, an inhibitor of PAF, did not have an effect on either stroma colonies or hematopoietic colonies at a concentration below IC_{90}. Sphingosine and Curcumin completely stopped the growth of stroma colonies and hematopoietic colonies even when the concentration was at IC_{50} for WT cells suggesting that these are highly toxic drugs (What conc was used?). Chelerythrine chloride was mildly toxic, which changed with increasing concentration. Our results indicate that MK-591, MK-886, and PCA-4248 could be good candidates for medical clinical trails.

Use of these inhibitors of different signaling pathways gives us a better understanding of the mechanism of action of the bioactive lipids in breast cancer. By blocking one of these pathways we were able to block the growth of breast cancer cells, especially the cells that develop drug resistance. However not all of these inhibitors serve as a potential therapeutic agents because of their toxicity to the bone marrow cells. The MK drugs which block 5-LO pathway and block cell growth, were not toxic to the bone marrow cells and therefore may be of some use in controlling the growth of breast cancer cells openning new avenues for therapeutic intervention.

Figure 1. Inhibition of proliferation in (A and C) WT or (B and D) ADR MCF-7 cultures with lipoxygenase inhibitors; (●) MK591, (■) MK886, (▲) NDGA for A and B; (▼) curcumin, (◆) ketoconazole, and (➢) AA881 for C and D. Values are expressed as % of the control. Symbols represent the mean ± S.E.M. of 3 determinations.

Figure 2. Inhibition of proliferation with PKC inhibitors in (A) WT and (B) ADR MCF-7 cultures; (●) sphingosine, (■) chelerythrine chloride. Values are expressed as % of the control. Symbols represent the mean ± S.E.M. of 3 determinations.

Figure 3. Inhibition of proliferation in (●) WT and (◆) ADR MCF-7 cultures with (A) PAF binding inhibitor PCA-4248 and (B) tumor promoter thapsigargin. Values are expressed as % of the control. Symbols represent the mean ± S.E.M. of 3 determinations.

Figure 4. Human bone marrow stroma colonies with one week ~~treatment after a~~ 2 weeks incubation. (A) The number of colonies, (B) average area of the colonies, (C) and the percentage of coverage of the colonies are shown. Each bar represents the mean ± S.E.M. of 3 determinations, with each determination consisting of 4 separate trials.

Figure 5. Human bone marrow stroma colonies with one week ~~treatment after a~~ 4 weeks incubation. (A) The number of colonies, (B) average area of the colonies, (C) and the percentage of coverage of the colonies are shown. Each bar represents the mean ± S.E.M. of 3 determinations, with each determination consisting of 4 separate trials.

Figure 6. Human bone marrow stroma colonies with continuous treatment ~~after a~~ for 2 weeks ~~incubation~~. (A) The number of colonies, (B) average area of the colonies, (C) and the percentage of coverage of the colonies are shown. Each bar represents the mean ± S.E.M. of 3 determinations, with each determination consisting of 4 separate trials.

Figure 7. Human bone marrow stroma colonies with continuous treatment ~~after a~~ for 4 weeks ~~incubation~~. (A) The number of colonies, (B) average area of the colonies, (C) and the percentage of coverage of the colonies are shown. Each bar represents the mean ± S.E.M. of 3 determinations, with each determination consisting of 4 separate trials.

Figure 8. The treatment of human bone marrow cells with drugs at different concentrations.

Table 1. The inhibitors of signal transduction pathways that are not effective for MCF-7 cells under the present conditions.

Name	Function of Inhibition	Concentration range
ETYA	Arachidonic Acid uptake, Arachidonic acid specific and nonspecific acyl-CoA synthetase, PLA2, cytochrome P-450, LO/CO	20-50μM
Phenidone	LO/CO	100-400μM
Aspirin	CO	10-30μM
Benzylimidazole	Induces cytochrome P-450 isozymes including lauric acid, Thromboxane A2 synthase	5-50μM
H7	PKA, PKG, MLCK, and PKC	10-30μM
Tyrphostin 1	Inactive tyrphotin	10-40μM
Tyrphostin 23	EGF receptor kinase activity	10-40μM
Gossypol	RBL-1,5-LO and 12-LO, PAF, and leukeotriene-induced guinea pag parenchyma contractions	20-50μM
NMDA	Excitatory amino acid	30-50μM

LO: Lipoxygenase; CO: Cyclooxygenase

WT ADR BAL

Table 2. Hematopoietic colony distribution

Inhibitors	BFU-E	CFU-E	CFU-GM	CFU-MIX	CFU-GEMM
control	30	43	55	13	4
MK591-16	24	24	70	14	3
MK591-40	37	26	63	12	3
MK591-70	10	16	24	0	0
NDGA-16	26	25	61	12	4
NDGA-40	4	4	18	1	0
NDGA-70	0	0	0	0	0
PCA-13	29	24	93	12	3
PCA-38	29	15	71	4	2
CHEL-10	26	28	83	21	4
CHEL-20	24	30	40	2	2
CHEL-40	11	18	0	0	0
MK-886-8	33	25	91	12	4
MK886-30	28	21	56	8	2

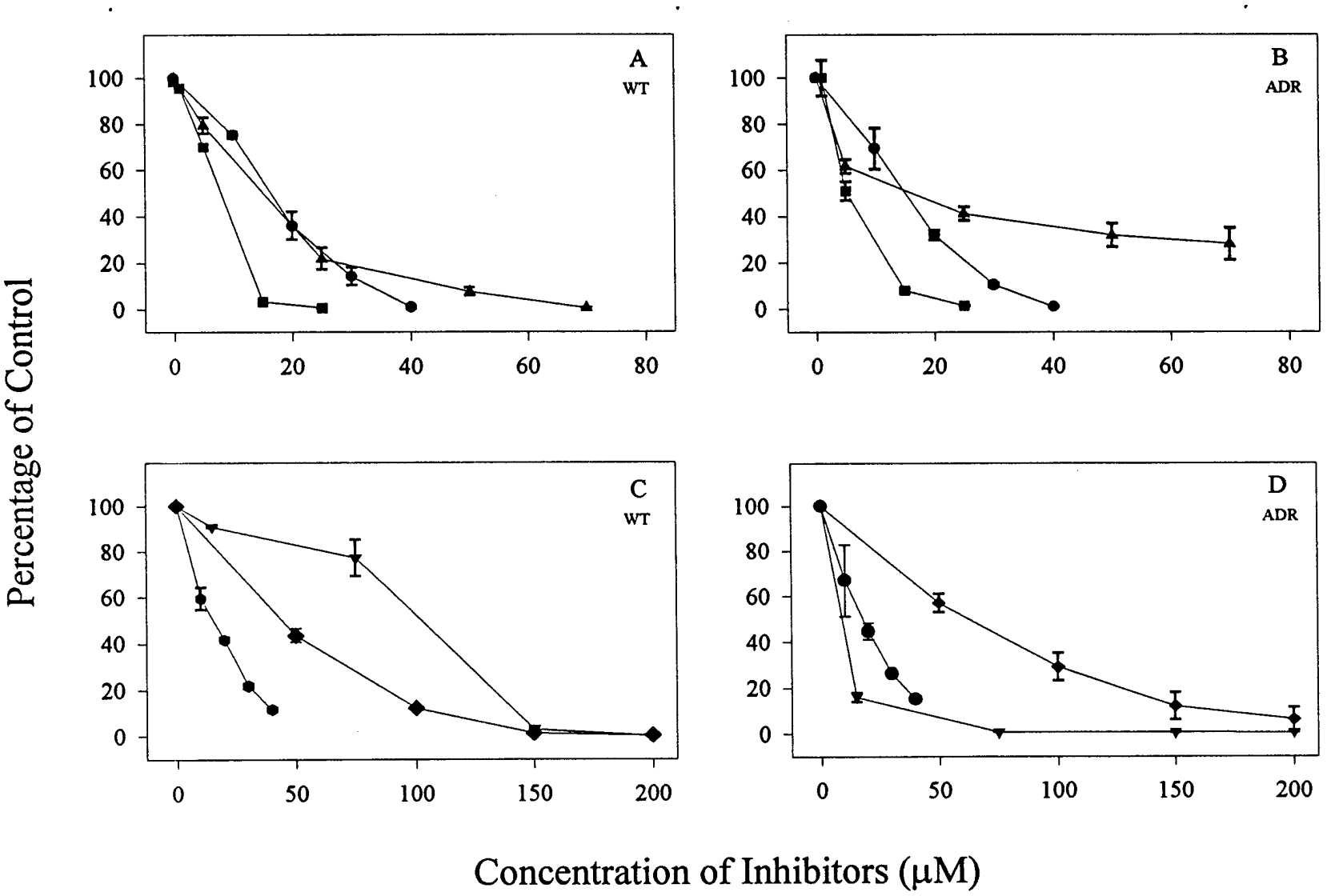

A
WT
B
ADR
C
WT
D
ADR
Percentage of Control
Concentration of Inhibitors (μM)

Figure 1.

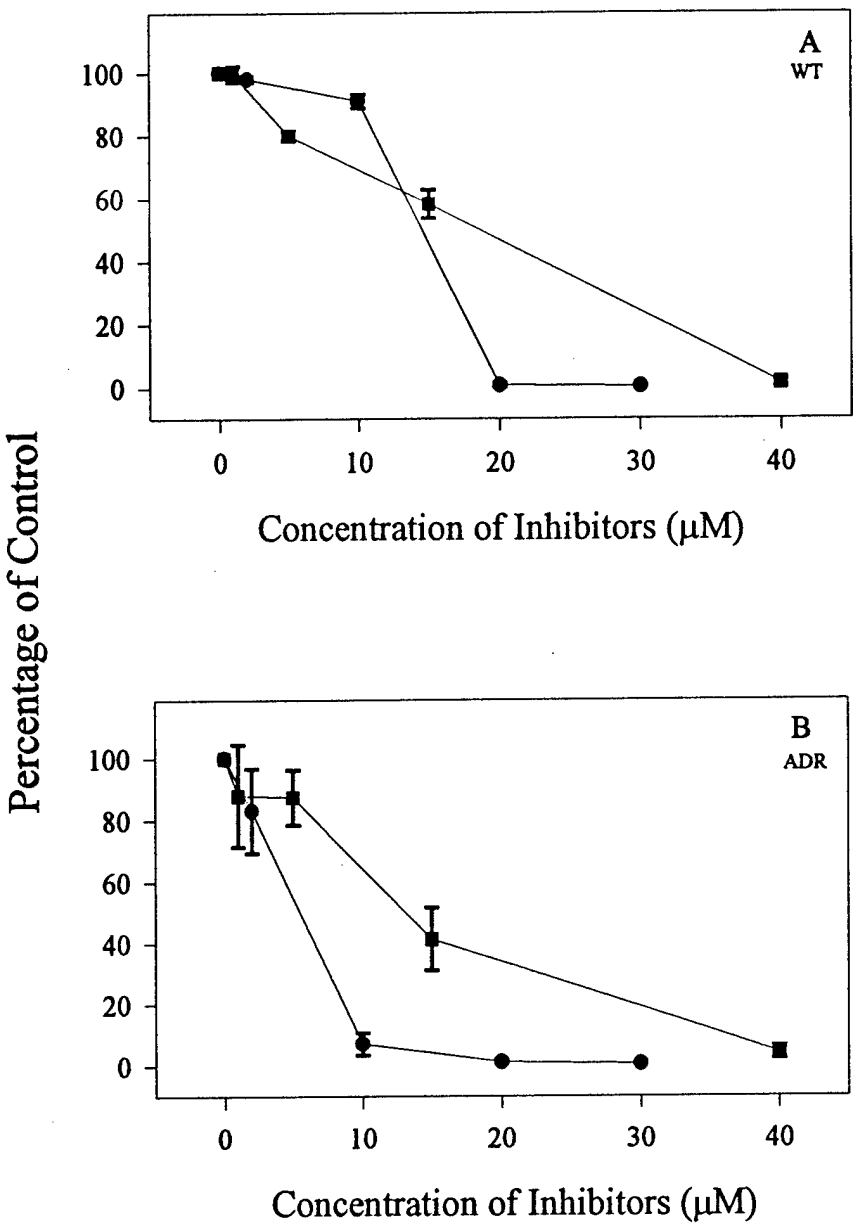

A
WT
100
80
60
40
20
0
0
10
20
30
40
Concentration of Inhibitors (μM)
Percentage of Control
B
ADR
100
80
60
40
20
0
0
10
20
30
40
Concentration of Inhibitors (μM)

Figure 2.

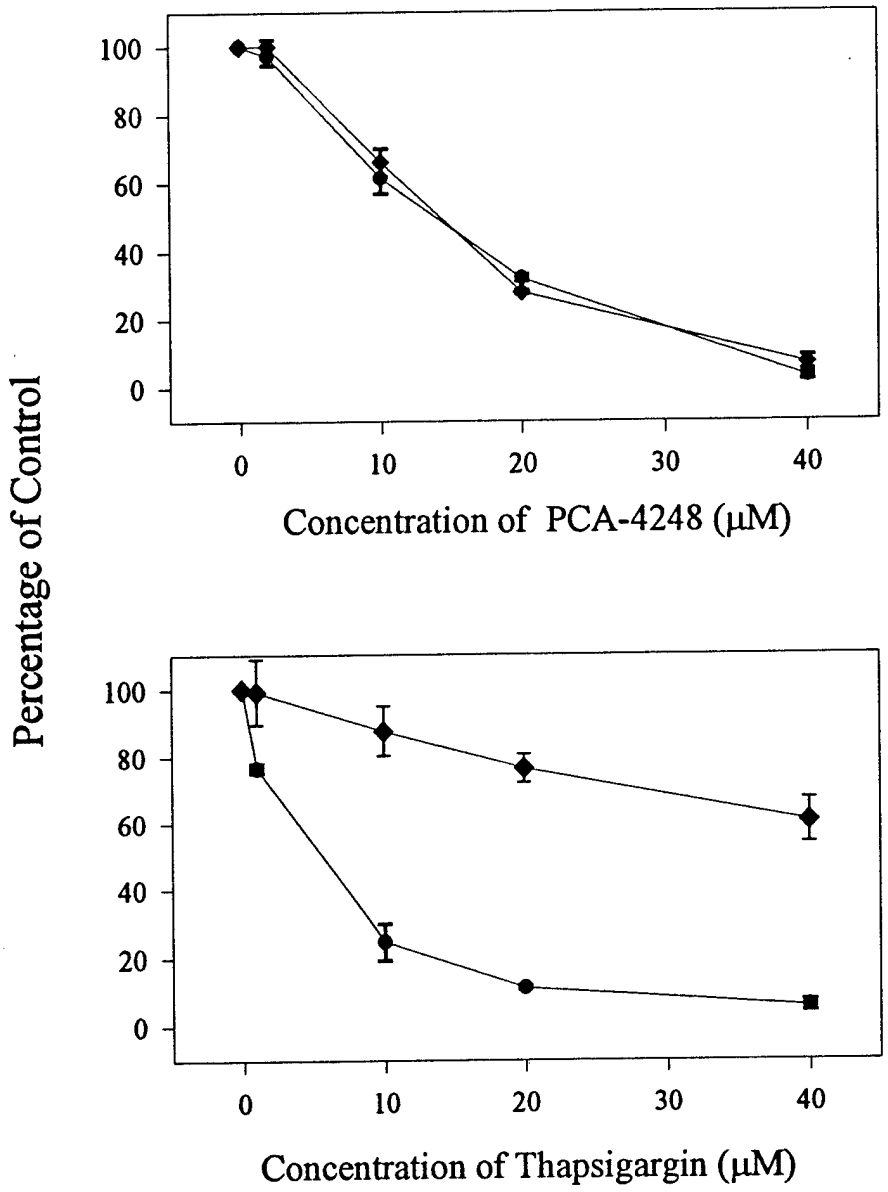

Percentage of Control
100
80
60
40
20
0
0
10
20
30
40
Concentration of PCA-4248 (μM)
100
80
60
40
20
0
0
10
20
30
40
Concentration of Thapsigargin (μM)

Figure 3.

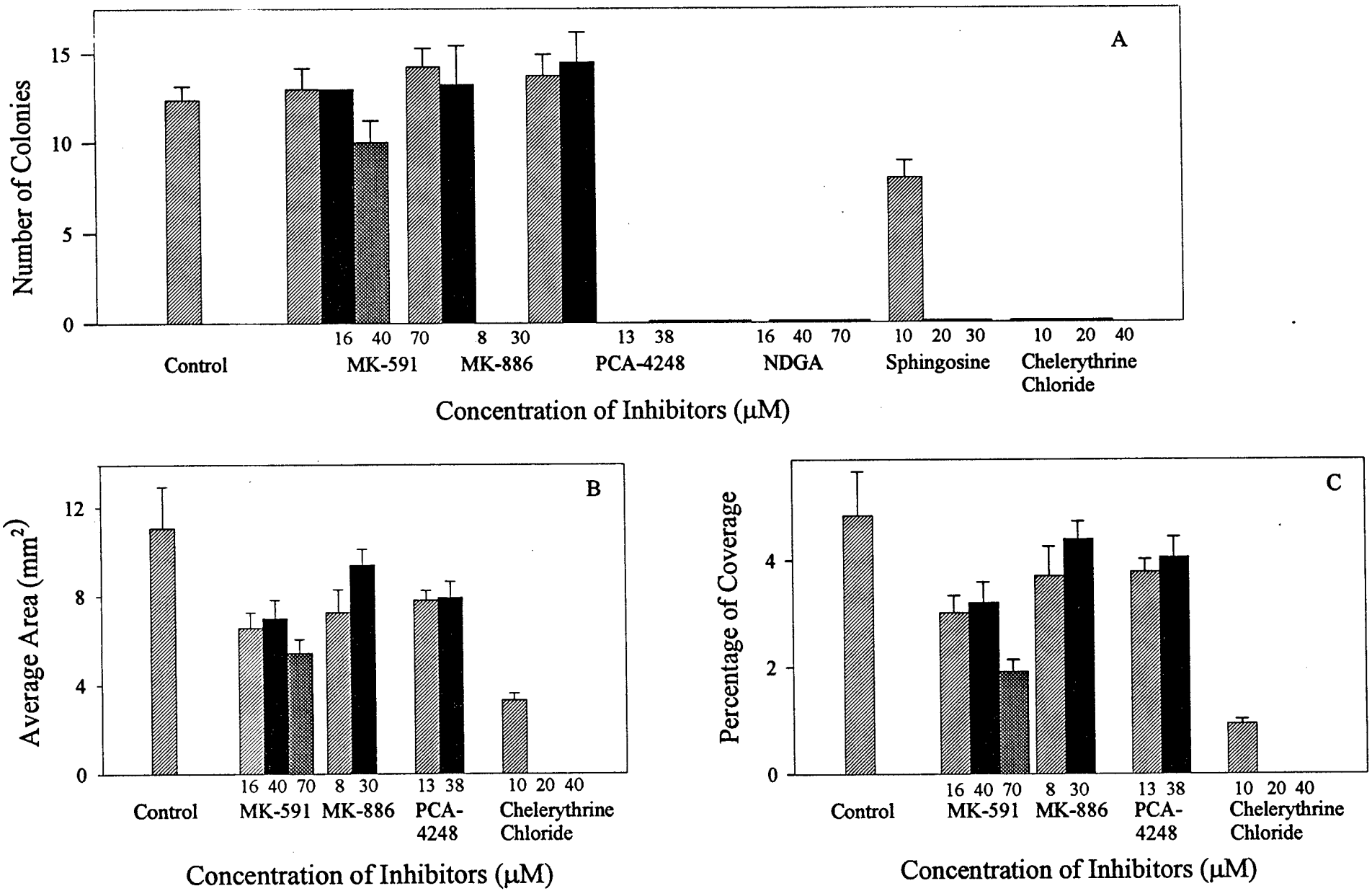
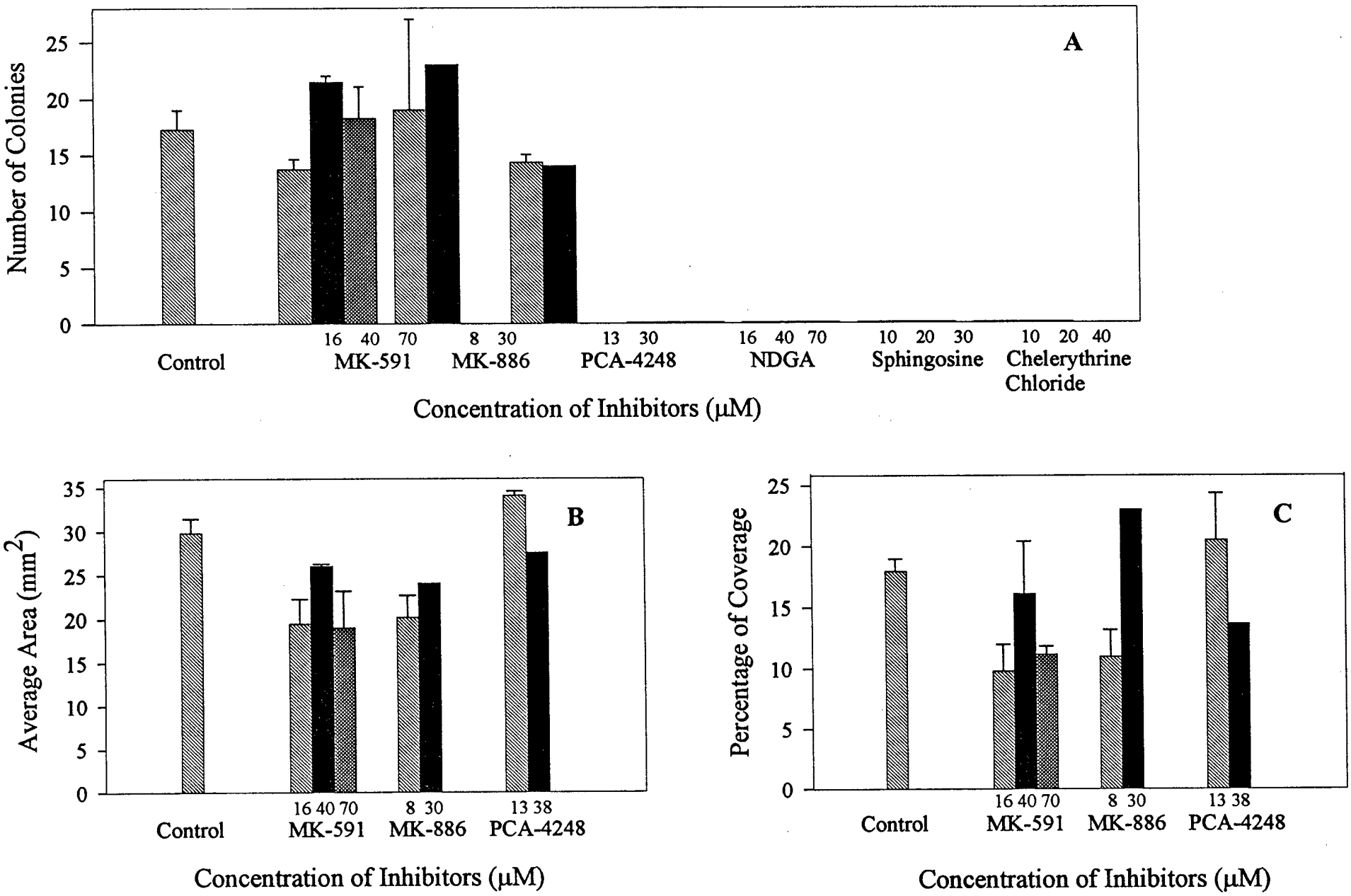

A
Number of Colonies
15
10
5
0
16 40 70
8 30
13 38
16 40 70
10 20 30
10 20 40
Control
MK-591
MK-886
PCA-4248
NDGA
Sphingosine
Chelerythrine Chloride
Concentration of Inhibitors (μM)
B
Average Area (mm²)
12
8
4
0
16 40 70
8 30
13 38
10 20 40
Control
MK-591
MK-886
PCA-4248
Chelerythrine Chloride
Concentration of Inhibitors (μM)
C
Percentage of Coverage
4
2
0
16 40 70
8 30
13 38
10 20 40
Control
MK-591
MK-886
PCA-4248
Chelerythrine Chloride
Concentration of Inhibitors (μM)

Figure 4.


A
Number of Colonies
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5
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15
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25
Control
16 40 70
MK-591
8 30
MK-886
13 30
PCA-4248
16 40 70
NDGA
10 20 30
Sphingosine
10 20 40
Chelerythrine Chloride
Concentration of Inhibitors (μM)
B
Average Area (mm2)
0
5
10
15
20
25
30
35
Control
16 40 70
MK-591
8 30
MK-886
13 38
PCA-4248
Concentration of Inhibitors (μM)
C
Percentage of Coverage
0
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25
Control
16 40 70
MK-591
8 30
MK-886
13 38
PCA-4248
Concentration of Inhibitors (μM)

Figure 5.

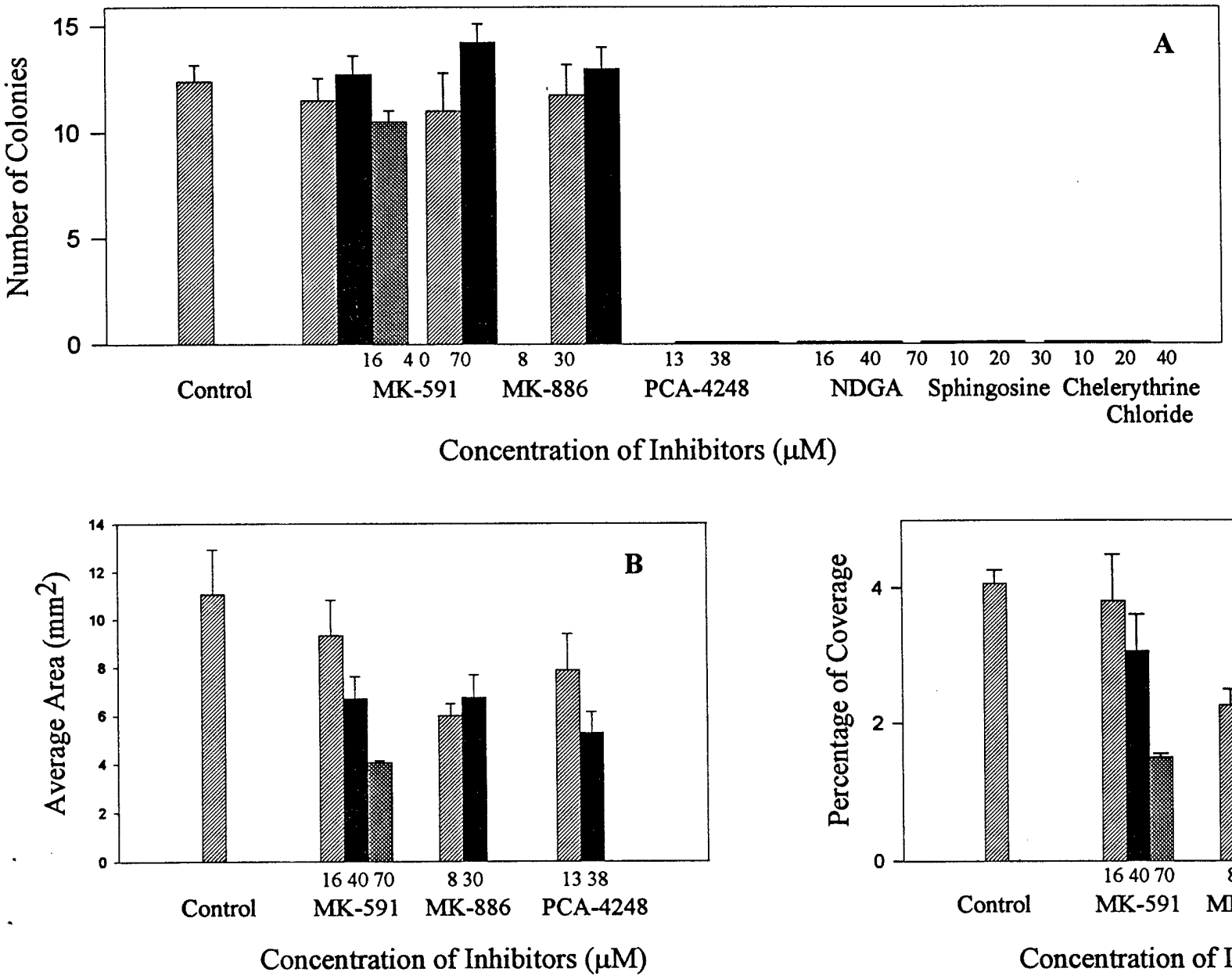
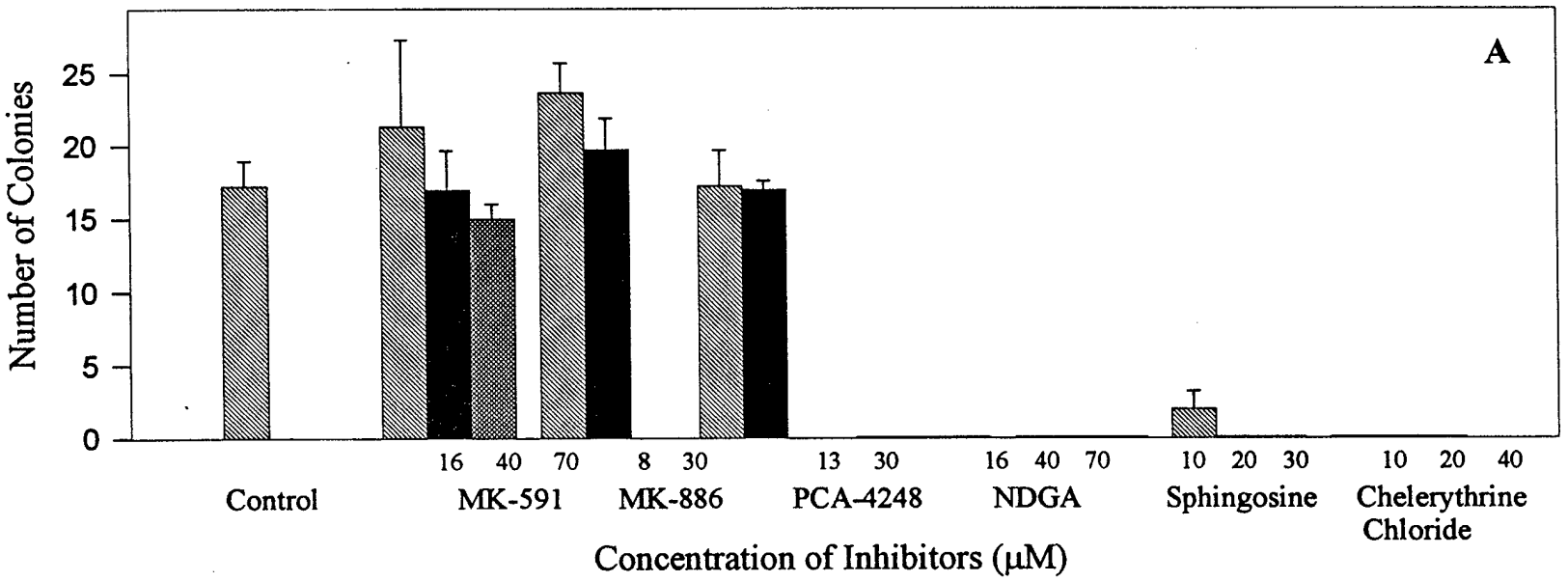
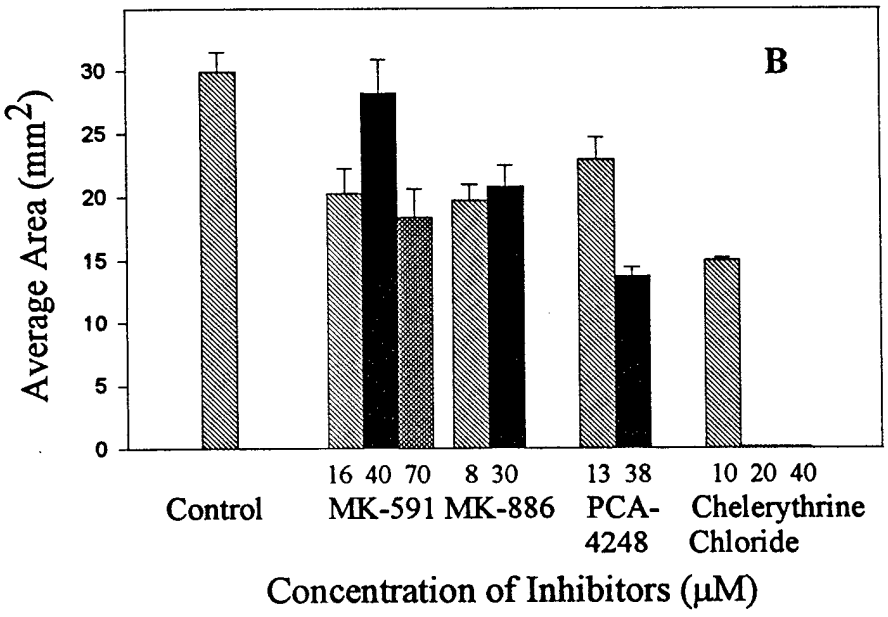

A
Number of Colonies
15
10
5
0
16 40 70 8 30 13 38 16 40 70 10 20 30 10 20 40
Control
MK-591
MK-886
PCA-4248
NDGA
Sphingosine
Chelerythrine Chloride
Concentration of Inhibitors (μM)
B
Average Area (mm2)
14
12
10
8
6
4
2
0
16 40 70 8 30 13 38
Control
MK-591
MK-886
PCA-4248
Concentration of Inhibitors (μM)
C
Percentage of Coverage
4
2
0
16 40 70 8 30 13 38
Control
MK-591
MK-886
PCA-4248
Concentration of Inhibitors (μM)

Figure 6.


A
Number of Colonies
25
20
15
10
5
0
16 40 70 8 30 13 30 16 40 70 10 20 30 10 20 40
Control
MK-591
MK-886
PCA-4248
NDGA
Sphingosine
Chelerythrine Chloride
Concentration of Inhibitors (μM)


B
Average Area (mm2)
30
25
20
15
10
5
0
16 40 70 8 30 13 38 10 20 40
Control
MK-591
MK-886
PCA-4248
Chelerythrine Chloride
Concentration of Inhibitors (μM)

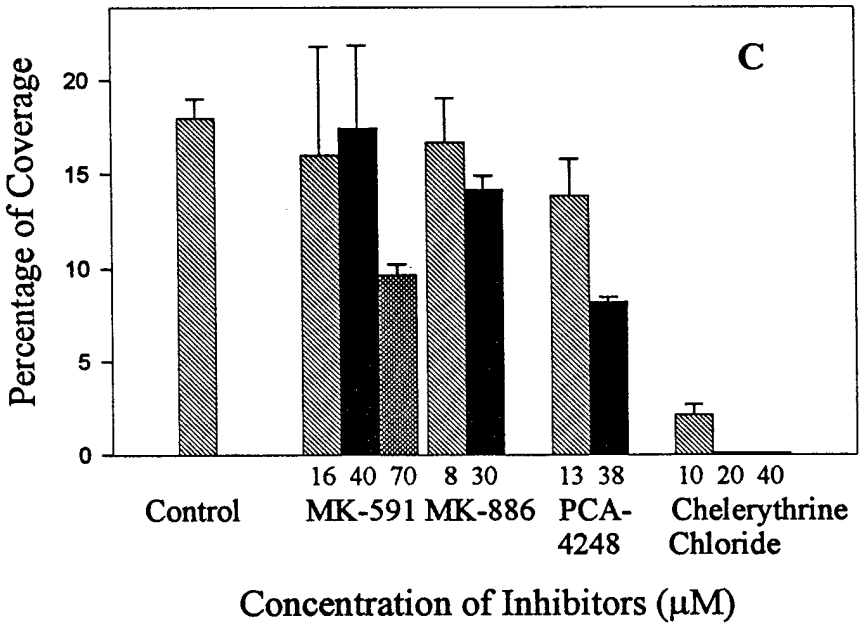
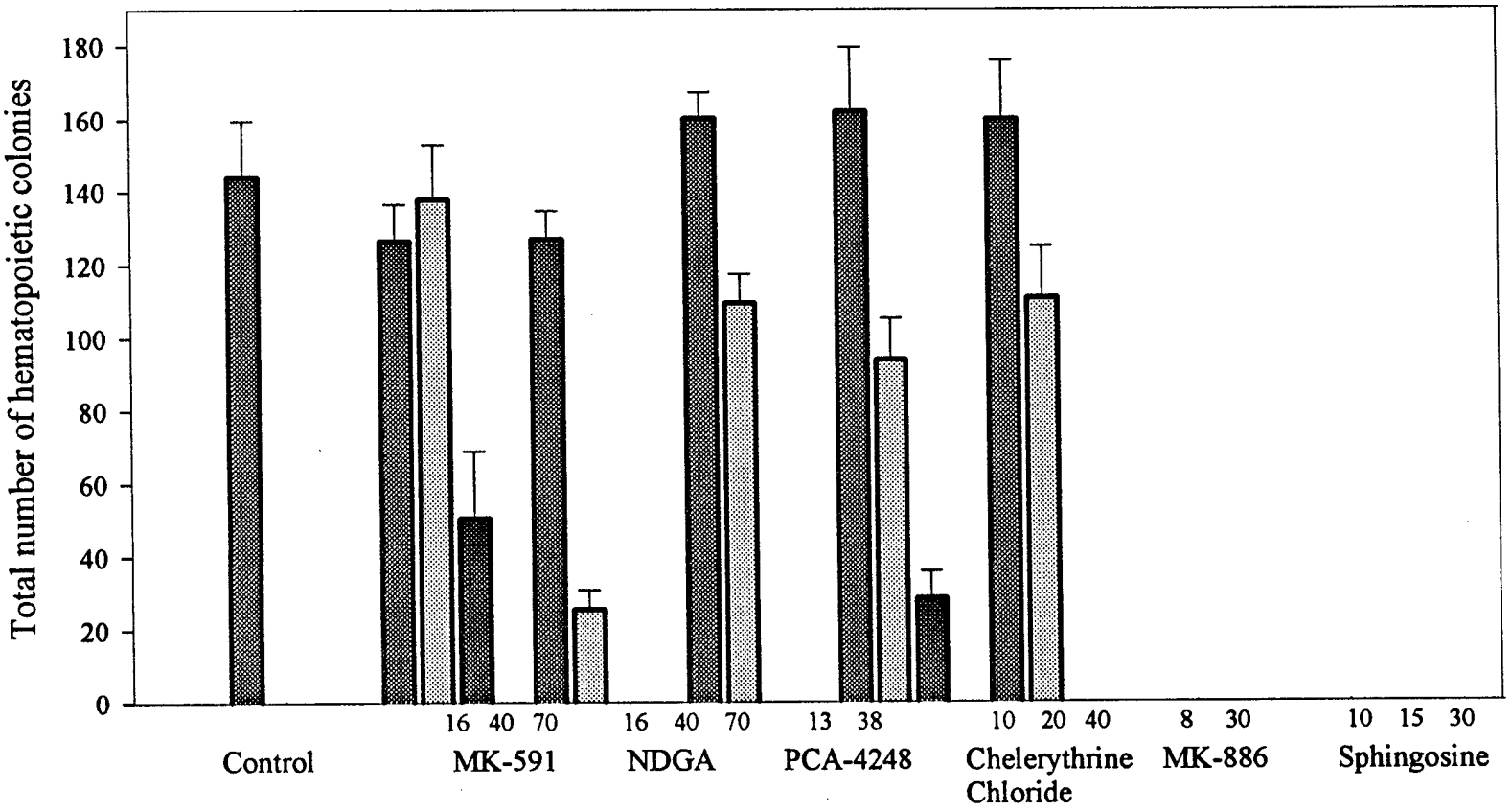

C
Percentage of Coverage
20
15
10
5
0
16 40 70 8 30 13 38 10 20 40
Control
MK-591
MK-886
PCA-4248
Chelerythrine Chloride
Concentration of Inhibitors (μM)

Figure 7.



The treatment of human bone marrow cells with drugs at different concentrations (μM)

Figure 8.

Treatment of MCF-7 breast cancer or human bone marrow cells with heteropolyanions

XiaoYan Zhang, Jonathan Leung, Rina Das, and Marti Jett. Walter Reed Army Institute of Research, Washington, DC 20307, and NCI, BMPB, Bethesda, MD.

Abstract: A group of compounds called heteropolyanions are free-radical scavengers that show excellent antiproliferative effects and do not induce drug-resistance as compared with doxorubicin. The IC_{50} of doxorubicin resistant MCF-7 cells are more sensitive to these heteropolyanions than normal WT MCF-7 cells. The treatment of synchronized ADR MCF-7 at G_1/G_0 with heteropolyanions causes cell death during DNA synthesis (S Phase). However, WT cell cultures start to die after 30 hours with the same treatment. Colony assays and advanced therapeutic studies with mice show that these compounds exhibit little toxicity to cultures of human bone marrow cells or mice, suggesting that they may be potentially useful as a form of human breast cancer therapy.

MATERIALS AND METHODS

Cells. The human breast cancer MCF-7 cells were obtained from ATCC. The cells were grown in Improved Modified Essential Medium (IMEM) containing 8% fetal bovine serum, 50,000 units/liter penicillin and 5,000 μg/L streptomycin at 37°C with 5% CO_2. Cells (0.5×10^6 cells/well with 8 mL IMEM in 4-well plates) were synchronized with thymidine (final concentration = 2mM) for 12 hours before they were treated with inhibitors in cell cycle studies.

Human bone marrow light density cells were obtained from Poietic Technologies Inc.

The stroma colonies were grown in IMDM-based Long Term Culture Media[5] (LTCM) containing 25% horse serum (Hyclone) with 5% CO_2 and 100% humidity at 37°C.

Methyl(H^3)-thymidine incorporation: Inhibition of proliferation in WT or ADR MCF-7 cultures was performed in 96-well plates. Cells were plated 15,000 cells/well in 0.2 mL IMEM culture fluid[1] and incubated overnight. Inhibitors or heteropolyanions were added (50 μL) to achieve the indicated concentration and incubated for three days. During the last 18 hours, Methyl(H^3)-thymidine (1μCi / well) was added. The cells were trypsinized and harvested using Packard Unifilter System. Then, 40μL of Packard Microscint 0 scintillation cocktail was added and the filter plates were counted using Packard Top-count.

Sample preparation for cell cycle studies: Cells ($0.6x\ 10^6$) were plated in 4-well plates with 8 mL IMEM and incubated overnight. In order to synchronize the cells, 200 μL thymidine solution (80mM) was added to each well and incubated for 12 hours. After 12 hours, the media containing thymidine was aspirated and washed with FBS-free media twice and 8 mL of fresh media was added to each well. The inhibitors (using stock solutions that were more than 250 times the desired concentration) were added immediately in the fresh media and then incubated for different time periods. The cells were incubated with 1X trypsin, harvested, and then washed with 1X PBS and IMEM. The remaining cell pellets, to be used in DNA staining, were stored in a -80 C freezer.

DNA staining: Cells were stained as described[1]. 0.5 mL of NIM buffer containing 50μg/mL and 1μL RNase was added to approximately 0.35 μL cell suspension. The

stained samples were incubated in the dark at room temperature for at least 30 minutes. Using a 47 μm nylon mesh, the stained nuclear suspension was filtered.

Flow Cytometry and Data analysis: P.I. stained samples were measured on a FACScan flow cytometer equipped with Double-discriminator. The instrument was aligned as described.[1] Linar red fluorescence was collected using a Cell-fit program at 50-150 events.

Arachidonic acid metabolism: MCF-7 cells (0.3 million cells/well) were plated in 4-well plates with 7 mL IMEM and incubated for 24 hours. In order to starve the cells, the original media was removed and replaced with 7 mL FBS-free IMEM media and incubated for 48 hours. ^{3}H Arachidonic Acid (100 μCI/well) was added to each well and the plates were incubated overnight. Then the media was aspirated, washed with 10 mL 1X PBS, and aspirated once more. A time course experiment was performed for 2 minutes and 2 hours. Two control wells (IMEM + 2mM Ca^{+2} and IMEM + 2mM Ca^{+2} + 2 μg/mL IGF) were performed for each time period. The heteropolyanion drugs were premixed with the solutions mentioned above and added to the cells. After the desired time period was reached, 25 μL of 22M Formic Acid and 25 μL of 10mM BHT were added to stop the reaction. The plates were immediately placed on dry ice. The cells were scraped, washed with acidified water, and transferred to 15 mL tubes for extraction.

Arachidonic Acid Extraction:

Sep Pak Preparation: Sep paks were attached to the appropriate size syringe and placed in a vacuum filtration apparatus. The sep parks were prepared for extraction by adding 4 mL methanol followed by 10 mL distilled water and vacuuming each through.

Sample Preparation: The samples were centrifuged at 1000 g for 10 minutes. The supernatant was transferred to a new set of tubes and the pellet was stored on dry ice. Internal Standard tubes were made by adding 2 mL saline (original sample volume) to 13 mL acidified water. If needed, the pH of the samples should be adjusted to 3.5-4.0. Approximately 50 µL of internal standard (made by adding 5 mL methanol + 500 µL 15 HEDE + 50 µL Eicosatrienoic Acid) should be added to each sample to read 70,000-80,000 DPM. Two aliquots of 300 µL were removed from each sample and placed into scintillation tubes and counted (duel $^{3}H/^{14}C$).

Sample Extraction: The samples were poured through each sep pak, washed with 4 mL of 2% ethanol, and promptly removed to prevent air from getting through. The sep paks were washed with 10 mL of a 85% Acetonitrile/15% Methanol solution and the solutions were collected in 15 mL polypropylene tubes. Then, the sep paks were washed with 10 mL of a 100% Methanol solution and the solutions were collected into their corresponding tubes.

HPLC Preparation: All of the tubes that were collected from the Acetonitrile phase were dried using the Speed Vac (-80 deg C), washed with methanol and dried again. 100 µL of methanol was added to each tube and the solution was transferred to HPLC vials for HPLC analysis.

Colony Assay: Human bone marrow stroma colony studies were performed using light-density marrow cells[2]. Cells ($2x10^5$ cells/well) were plated in 4-well plates with 5 mL LTCM and drugs were added (according to their desired concentration) on the second day. The media was changed with fresh media either with (continuous treatment) or without drugs (pulse) every week. The stroma colonies were stained on the second and fourth week of treatment with HEMA 3 (differential hematology stain) and the number and size of the colonies were measured.

The hematopoietic progenitor colony assay was performed using Methylcellulose-based Colony Cocktail from Stem Cell Technologies, Inc. (HCC-4434). Small volumes of drugs (20-50μL) were premixed with the cocktail (4.5 mL) and 0.5 mL of $7.0x10^5$ cells/mL were added. The cells in the cocktail (either with or without drugs) were plated in a 35mm diameter gridded tissue culture plate (Nunc. Inc.). After a two week incubation period, the hematopoietic colonies were counted using an inverted phase microscope with 40X magnification. Using the standard criteria from Stem Cell Technologies Inc. (Atlas of Human Hematopoietic Colonies), the colonies were classified into categories that included: CFU-GM, BFU-E , CFU-E, CFU-Mix, and CFU-GEMM.

Both stroma colony assay and hematopoietic colony assay experiments were performed with four replicates and repeated three times with different lots of cells. Regardless of the lot of the cells, the same results occurred.

Preliminary injections were given to groups of mice to determine useful dosage for experimentation. The doses used were .05mg/mouse, .2mg/mouse, .3mg/mouse, .4mg/mouse, .5mg/mouse, .6mg/mouse, and .7mg/mouse.

a) Dose response of mice to Sm-HPA: Different amount of Sm-HPA was injected into 5 mouse in each group. The injection with 5.5mg or 3mg in 1mL for each mouse causes mouse death after 4 or 5 day injection. For the injection with 1.8mg or 1mg in 0.5 mL for each mouse, the former causes mouse death after two week injection and the later only showed hair lose after one week.

b) Treatment with chronic doses for therapeutic window: Ten groups of 7 mice were injected with Sm-HPA in 0.25 mL 0f 1:1 injection water / vingeral lactose injection fluid. The number of injections and the behavior of the mice were shown in Table 4. The average weights during the injection time period was shown in Figure 4.

c) Tungsten distribution in mouse organs: Mice were injected 2mg/mouse and necropsied at 2h, 4h, 6h, 8h, 16h, 24h, and 48h. The separate organs were digested and the measurement of W were shown in Table 5. In order to examine the digestion of W, the organs were collected at three different treatment with 2.1mg of Sm-HPA a) Inject 0.7mg/mouse each week for three weeks; b) Inject 0.7mg/mouse each week for three weeks and keep the mice for 7 more weeks; c) Inject 0.2mg/ mouse each week for ten weeks.

d) Pathology observation:

CONCLUSIONS

Observations and Results

In order to determine the amount of heteropolyanions that could be injected over a period of several weeks, preliminary injections were given to individual mice. The first mice was given 5.5mg and died on the fourth day. The second mouse, given 3mg, also died on the fourth day. The third mouse was given two injections of 1.8mg each, a week apart, and

died after the second injection. The fourth mouse was given a 1mg injection. Some hair loss occurred, though no other changes were observed.

The Observations of the experimental groups are tabulated in Table X.

After one injection, differences in the skin tightness of mice injected with high amounts of the heteropolyanions was immediately apparent. The .3mg/mice group had slightly tighter skin while the groups which received injections of heteropolyanions between .4mg/mice and .7mg/mice had significantly tighter skin. After three weeks, injections were discontinued in the groups receiving between .5mg/mice and .7mg/mice as the skin became too tight. Skin in groups receiving lower amounts of heteropolyanions tightened more gradually. Skin tended to loosen once injections were discontinued.

Significant hair loss began to occur in the groups receiving high amounts of heteropolyanions during weeks 4 through 7, after injections had ceased. The .7mg/mice group lost as much as ½ to ¼ of total body hair and the .6mg/mice group lost approximately ½ total body hair. Some hair recovery was later observed. Slight hair loss also occurred in the .4mg/mice group during weeks 6 and 7 and in the .3mg/mice group during weeks 8 through 11.

The average weight of the mice in the control and experimental groups is shown in Figure Y. For groups receiving low amounts of heteropolyanions, normal weight gain occurred for the first few weeks, with minor weight loss later on. In the .4mg/mice group, the weight loss was significant. With groups receiving larger injections, a dramatic weight loss was observed between weeks 3 and 4, with most weight regained after injections were discontinued.

CONCLUSIONS

1. The treatment of MCF-7 breast cancer or human bone marrow cells with

heteropolyanions

2. Proliferation data: 1 figure and 1 table; (Drug resistant test)

3. Colony assay

4. Cell cycle data: a) synchronized at G1 phase; b) synchronized at G2 phase.

5. Arachidonate metabolism.

6. Mice experiment.

Two groups of mice were given .7mg/mouse injections. One group was euthanized when injection was discontinued after the third week. The second was euthanized after ten weeks. Both groups were (necropsied?) and the concentrations of heteropolyanions measured in different parts of the body. Other groups were also (necropsied?) after ten weeks.

The .4mg/mouse group and the .5mg/mouse group were not euthanized nor given injections for a period of two months (?). After two months, injections were again attempted; however, the skin was too tight to administer injections, so heteropolyanions were administered orally.

a) Toleration of heteropolyanion for single dose.

RESULTS AND DISCUSIONS

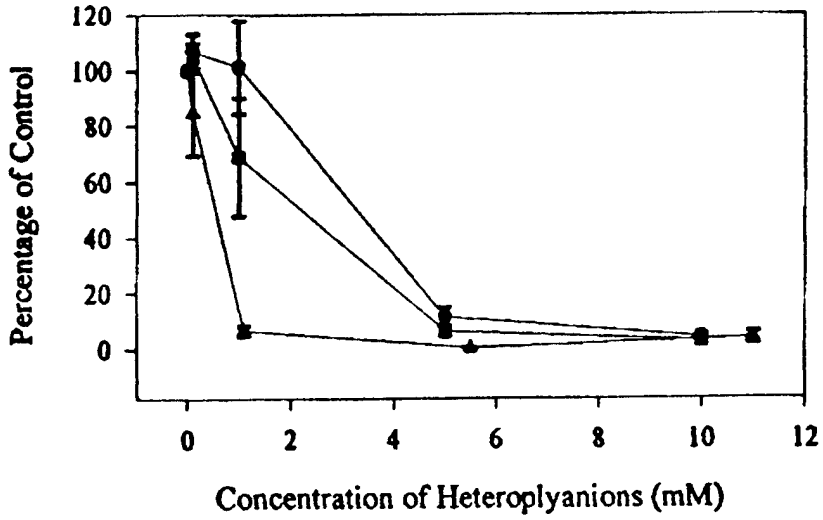
Inhibition of Proliferation were examined with heteropolyanions of group A, B, and C (Figure 1) in WT or ADR MCF-7 Cultures. Group A, [P5 W30 XO110]-m (X=) showed similar effective antitumor activity despite different charges. The typical data were shown in Figure 2 and the rest of them were shown in Table 1. It shows clearly that ADR MCF-7 cell cultures are more sensitive to these HPA than WT MCF-7. Almost all of IC50 for ADR cells are lower than these for WT cells. The group A polyanions The groups B and C do not show significant antitumor activity under present conditions (concentration 1-60μM). This may indicate that the size or the shape of the heteropolyanion are key factors in regards to biological activities.

The toxicity studies of the heteropolyanions to human bone marrow cells

Hematopoitic colonies of human bone marrow monnulear cells were treated with different concentrations of [SmP5 W30 O110]12- and [NaP5 W30 O110]14- to test for toxicity. The total number of colonies remaining after treatment are shown in Figures 3-6. The heteropolyanions were used in concentrations of 1mM, 6 mM, and 12 mM. As the concentration increases the total of the colonies decrease. The total of the colonies remain as much as 1/3 of control even at very high concentration of HPA (12μM).

The distribution of deferent colonies are tabulated in Table Y. The toxicity of the heteropolyanions was evident in the BFU-E and CFU-E colonies at 6μM. Both heteropolyanins did not have much effect on the CFU-GM colonies. All colonies of BFU-E and CFU-E were diminished and that of CFU-CM was slightly decreased at 12 mM.

Mice experiment:


Percentage of Control
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Concentration of Heteroplyanions (mM)

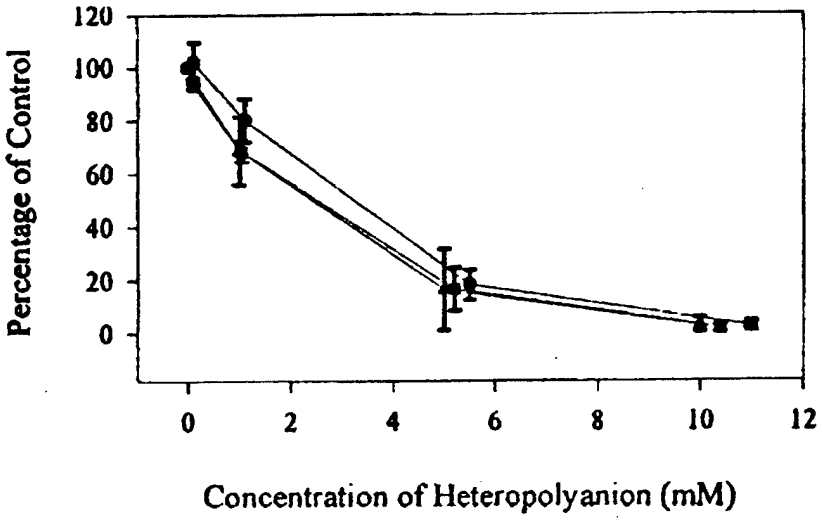

Percentage of Control
120
100
80
60
40
20
0
0
2
4
6
8
10
12
Concentration of Heteropolyanion (mM)

Figure 1.

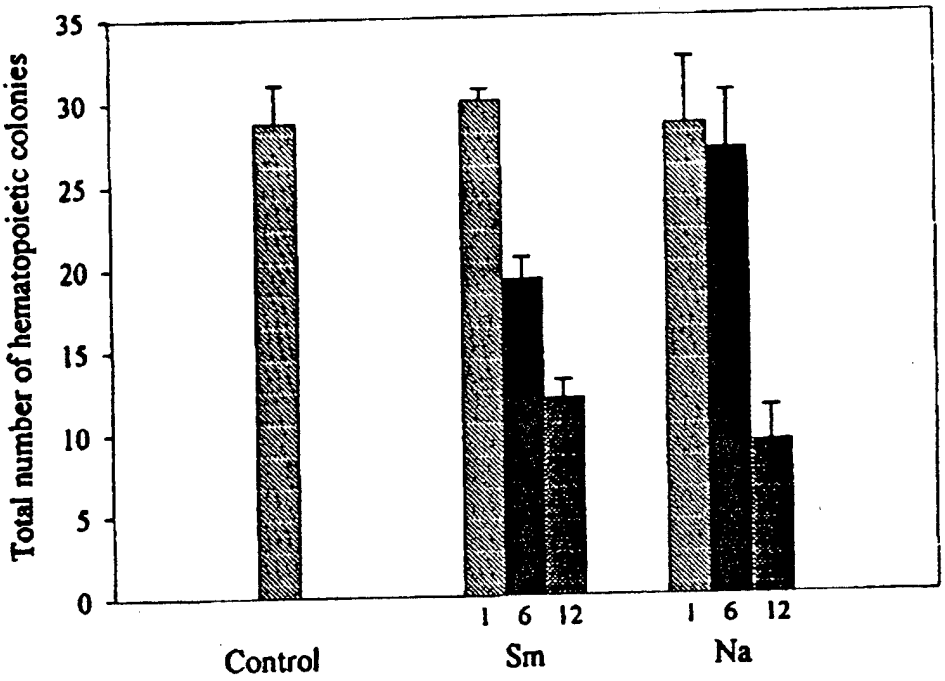
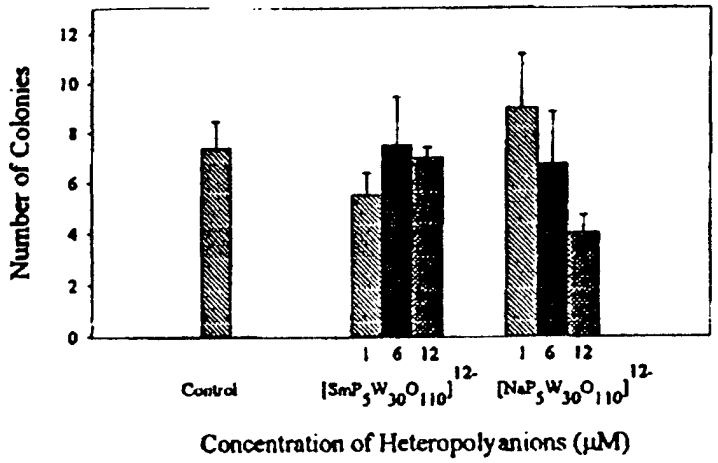
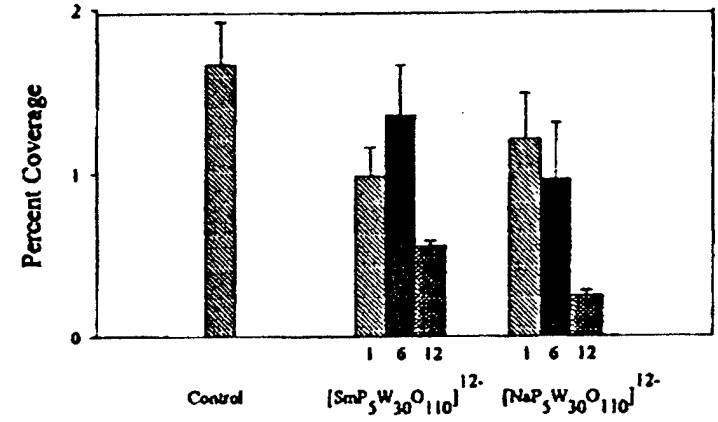

Total number of hematopoietic colonies
35
30
25
20
15
10
5
0
1 6 12
1 6 12
Control
Sm
Na

Figure 2.





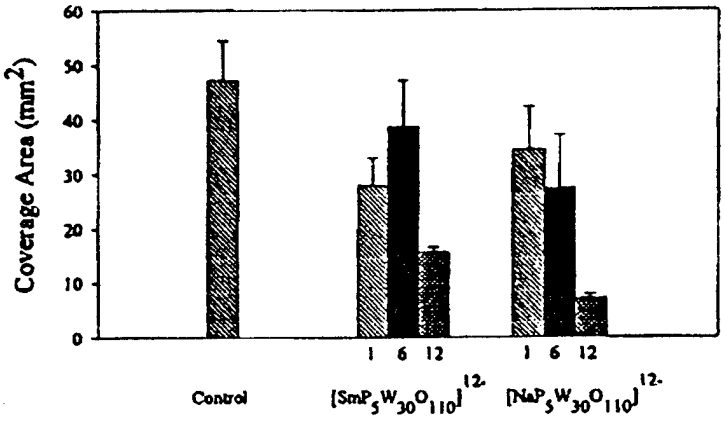
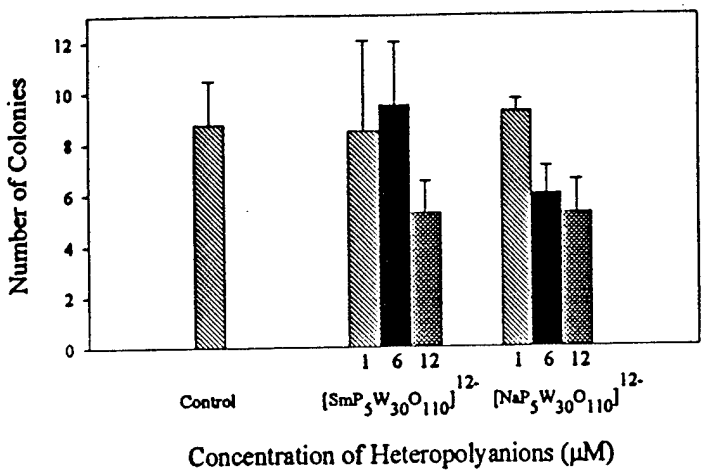
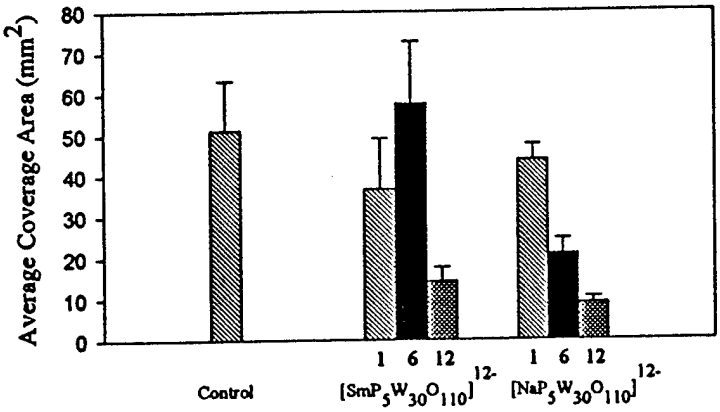


Figure 3. 2 weeks continuous





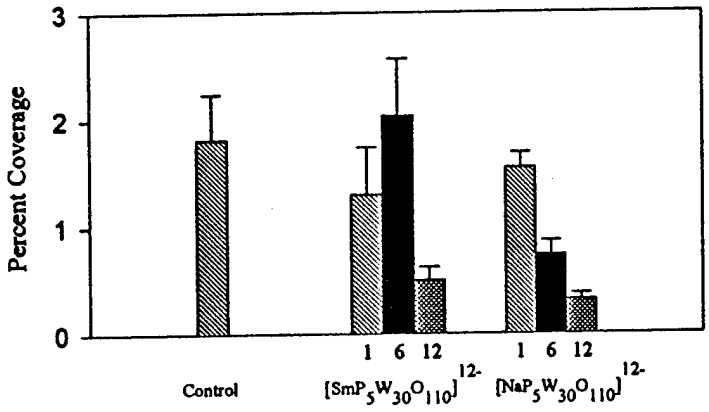
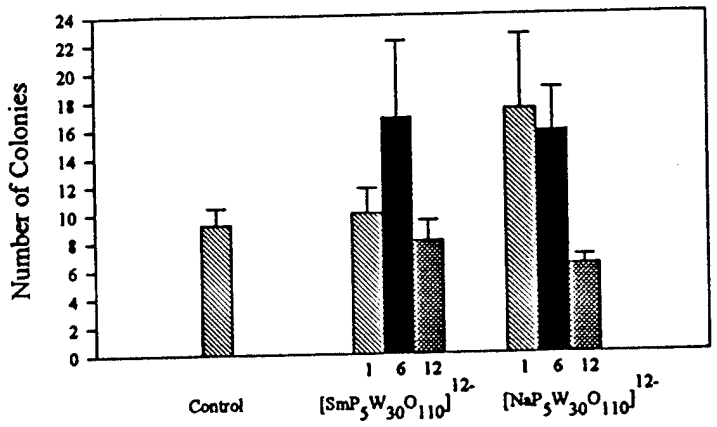
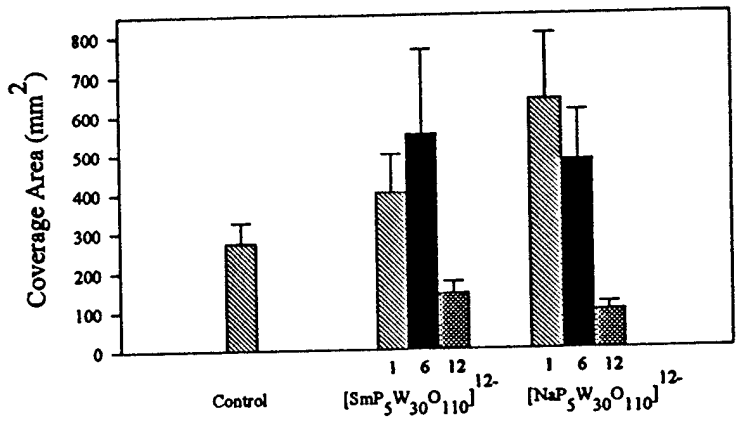


Figure 4. 2 weeks pulse





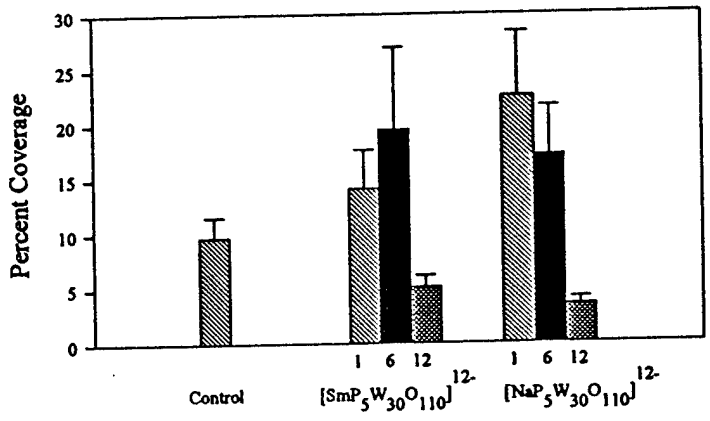
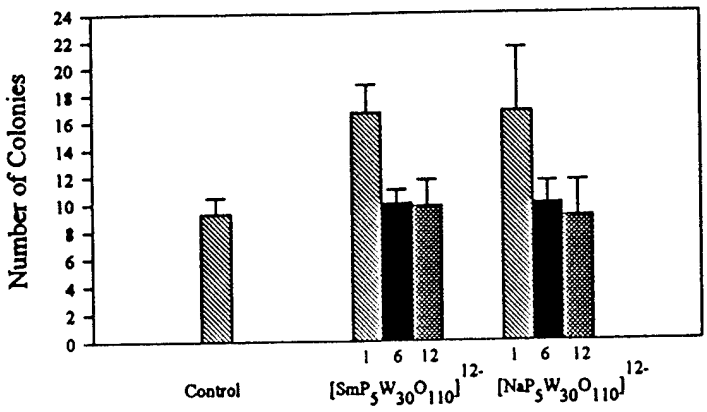
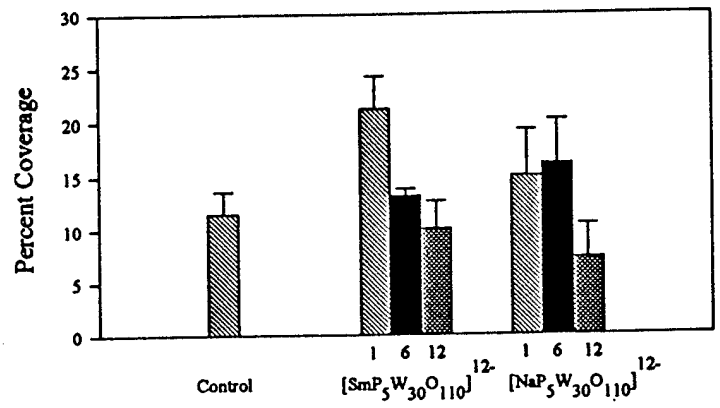


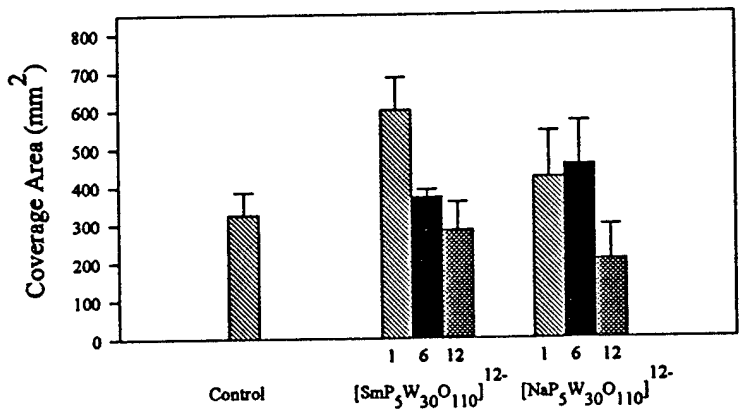
Figure 5. 4 weeks continuous



Concentration of Heteropolyanions (μM)

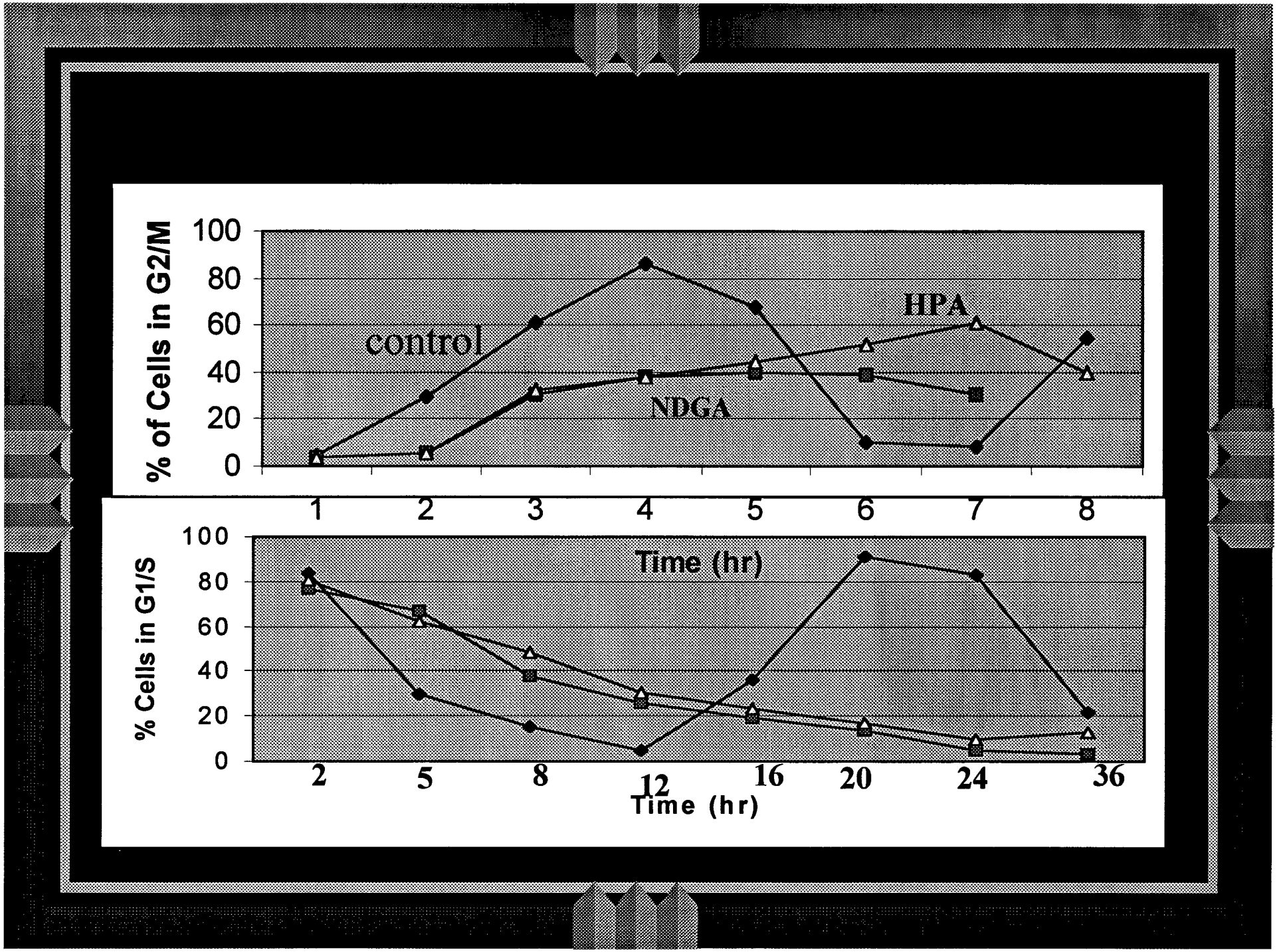


Concentration of Heteropolyanions (μM)



Concentration of Heteropolyanions (μM)

Figure 6. 4 weeks pulse


% of Cells in G2/M
100
80
60
40
20
0
control
HPA
NDGA
1
2
3
4
5
6
7
8
% Cells in G1/S
100
80
60
40
20
0
Time (hr)
2
5
8
12
16
20
24
36
Time (hr)

CROSS TALK BETWEEN MAP KINASE PATHWAY AND ARACHIDONIC ACID PATHWAY IN THE SIGNALING CASCADE OF IGF-1 IN BREAST CANCER CELLS.

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ABSTRACT

Insulin Growth Factor I /(IGF-1) when bound to its receptor, initiates a molecular signaling cascade that ultimately results in cell growth. Two of the many pathways involved in the growth of breast cancer cells are the Arachidonic Acid /(AA) pathway and the Ras-MAPK pathway. The former involves the release of AA from the cell membrane in response to IGF-I. The cyclooxygenase pathway and the lipoxygenase/(LO) pathways produce eicosanoid metabolites which are known to stimulate the growth of cancer cells. The MAP pathway deals with autophosphorylation of the IGF-1 receptor and the subsequent activation of ras, raf, and ultimately MAP kinase; MAPK activates various transcription factors leading to cell division. In the present study, we have analyzed the role of raf, MEK, MAP Kinase and 5-LO in the signaling of IGF-1 in human breast cancer cells /(578T). Raf, MEK and MAP Kinase were phosphorylated within 1 minute of IGF-1 treatment, reaching its peak by five minutes. Tyrosine phosphorylation of 5-LO was observed within 1 minute of IGF-1 stimulation. These results suggest that both signaling pathways are activated in response to IGF-1 in human breast cancer cells. To resolve the exact role of each of these kinases and establish a possible cross talk between the signaling pathways, we used several known inhibitors of these pathway. By blocking each of the pathways at different points, we should be able to pinpoint the exact relationship between the two cellular signaling pathways. The results suggest that there is cross talk between the two signaling pathways. Further studies are under way to determine the exact relationship.

BACKGROUND:

Although screening mammography and the increased use of breast conserving surgery and adjuvant chemotherapy have improved the quality of life and prolonged survival for women with breast cancer, additional therapeutic strategies are needed to combat the disease. Various studies

have suggested dietary fat content, especially polyunsaturated fatty acids, promotes tumor growth by increasing synthesis of eicosanoids, particularly AA products (Wynder et al. 1986; Welsch and Aylsworth, 1983, Carter et al. 1983). The possible role of arachidonic acid derived eicosanoids as regulators of neoplastic cell growth is an area of significant interest in breast cancer biology.

Activation of AA metabolism is initiated by the release of AA from the phospholipid pool by the enzyme phospholipase A2 (Axelrod et al. 1988). AA is metabolized through the cyclooxygenase pathway which results in prostaglandins production (Boyle et al. 1994) or through the 5-lipoxygenase (5-LO) pathway, which results in the production of leukotriene (Henderson, 1994). Both prostaglandins and leukotriene directly stimulate the growth of malignant cells (Lee and Ip, 1992; Snyder et al. 1989). Agonist stimulation of cells initiates the translocation of enzymatically active 5-LO from a soluble site to a membrane bound site where it interacts with 5-LO activating protein (FLAP). FLAP is an 18 kDa membrane protein required in peripheral cells for the activation of 5-LO which results in the synthesis of the leukotriene LTB4 (Rouzer et al. 1990; Miller et al. 1990; Woods et al. 1995).

Compounds which preferentially inhibit AA metabolism are emerging as potentially important tools for cancer management. Although most experiments so far have focused on inhibitors of the cyclooxygenase pathway, there does not appear to be a direct correlation between the inhibition of cyclooxygenase activity and the inhibition of tumor growth (Fulton, 1984; Feldman and Hill, 1985; Noguchi and Ohta, 1993). Inhibitors of 5-LO metabolism have shown promise in the treatment of asthma, and shock (Larsen and Acosta, 1993; Henderson, 1994). Selective antagonists of 5-LO metabolism significantly reduce growth by a number of lung cancer cell lines (Avis et al. 1996). Linoleic acid stimulates tumor cell proliferation in vitro. In MDA MB-231 cells, inhibitors of the cyclooxygenase pathway and the lipoxygenase pathway were able to inhibit thymidine incorporation in these cells (Earashi et al. 1996). Recently it has been shown that proliferation of MCF-7 human breast cancer cells was blocked by specific inhibitors of bioactive lipids such as MK591, MK886 and AA861 (Zhang et al. 1996). In malignant transformed, prostate cells, a 5-LO inhibitor caused inhibition of proliferation indicated by decrease in DNA synthesis (Anderson et al. 1994). Tyrosine phosphorylation of 5-LO is important for its activation and translocation to the membrane fraction in HL-60 cells. Lepley et al. (1996) reported that tyrosine kinase inhibitors blocked the activation of 5-LO, its association with FLAP and subsequent formation of 5-HETE by A23187 stimulation of HL-60 cells. Several of the 5-LO inhibitors are in clinical trials for treatment of acute asthma, adult respiratory distress syndrome and arthritis. The exact mechanism of action of these inhibitors is still not clear, especially the signal transduction pathways.

Multicellular organisms have developed highly efficient regulatory networks to control cell

proliferation. The mitogens interact with their receptors and induce activation of a series of kinases which ultimately lead to an increase in gene expression and growth. Many of these mitogens activate *ras* which binds GTP and in turn activates *raf* and MAP kinase kinase (MEK). A common pathway for many mitogens also includes activation of MAP kinase which in turn is known to activate a wide variety of target proteins including transcription factors which control gene expression (Cobb et al. 1991; Nishida and Gotah, 1993; Frost et al. 1994). Tyrosine kinase inhibitors have been shown to block the growth of breast cancer cells by affecting the *ras* -MAP kinase signaling pathway (Das and Vonderhaar, 1996, Clark et al. 1996). Somatostatin signal transduction through its receptor SSTR4 involves simultaneous stimulation of AA metabolism and MAP kinase phosphorylation cascade through PTX-sensitive G proteins (Bito et al. 1994). SSTR4 activates MAP kinase and induces phosphorylation of the 85 kDa cytosolic phospholipase A2 in a PTX sensitive manner (Sakanaka et al, 1994). In addition the coexpression of 5-LO and FLAP has been demonstrated in transmembrane signaling of somatostatin receptor in hippocampal neurons (Lammers et al 1996). These signal transduction pathways shared by different mitogens may provide an efficient approach to accomplish clinically significant control of breast cancer.

In the present study we analyzed the effect of IGF on the growth of normal and a transformed breast cell lines, in relation to its effect on 5-LO and FLAP. We used a number of inhibitors of arachidonate metabolism to manipulate the outcome of agonist/antagonist effects in breast cancer. To test whether FLAP is physiologically functional and involved in IGF modulation of breast cancer cells, we used the compound MK-886, a potent and selective FLAP inhibitor. Also the effect of these inhibitors, along with tyrosine kinase inhibitors on various target signaling molecules such as MAP kinase, raf and MEK along with 5-LO was studied. If we can block the signaling pathways of breast cancer cells, it may lead to arrest of their uncontrolled growth, which ultimately could be of clinical significance.

MATERIALS:

Cell lines: Hs 578Bst- a normal breast cell line, and Hs 578T- a tumor cell line derived from ductal carcinoma of breast will be utilized. Both are estrogen receptor negative and will be obtained from ATCC, Rockville, MD.

Inhibitors: MK886, MK591, AA861, Thapsagargin, Wortmannin, tyrphostin, herbimycin, genestein will be obtained from Biomol, PA and PD 098059 from Parke-Davis, MI.

Antibodies: Antibodies for MAP kinase, PI3 kinase, cyclin D, cyclin E, cdk4, cdk6 will be obtained from Transduction Laboratories, Lexington, Kentucky.

METHODS:

Cell Culture: Cells were maintained routinely in Dulbecco's modified Eagle's medium supplemented with serum and growth factors.

Extraction and Western Blot

Hs 578T Cells were obtained from ATCC, IGF-1 was obtained from Intergen. Inhibitors were obtained from Biomol and Merck Frost.

Human breast cancer cells, (Hs578T), were serum starved for 48 hours and were treated with Insulin, IGF-1 or inhibitors and incubated for five minutes. Cells were then washed with 1X PBS and homogenised in lysis buffer containing 20 mM Hepes, 40 mM beta-glycerophosphate, 10 mM EGTA, 1 mM DTT, 150 mM NaCl, 1% NP40, 0.5% Sodium Deoxycholate, 1mM Sodium Orthovanadate, 1mM PMSF, 10ug/ml Aprotinin, 10ug/ml Leupeptin. Equal amounts of protein was loaded on a 10% SDS-Gel or immunoprecipitated and then resolved on a gel. Western blots were probed with specific kinase antibody and was detected using the ECL detection kit.

Release of arachidonic acid: Arachidonic acid release will be measured as described previously (Bito et al. 1994). Cells will be prelabelled with 3H-arachidonic acid in serum free medium. The cells will be washed and after various treatments, aliquots of the medium will be tested for the release of radioactive AA.

5-LO assay: Cytosol and nuclear fractions was prepared from cells treated with growth factors and inhibitors. These cell fractions were subjected to immunoprecipitation with anti-5LO antibody (Merck-Frost, Canada), resolved on a SDS-gel and after transfer to nitrocellulose probed with phosphotyrosine antibody.

Reverse transcriptase - polymerase chain reaction (RT-PCR) analysis: Poly (A) mRNA was prepared from the treated normal and breast cancer cells using Trizol method. Reverse transcriptase reaction was performed and PCR carried out using specific primers according to the method of Dixon et al. 1990. PCR products were resolved on an agarose gel and the southern blot was probed for specific oligo probes for FLAP and 5-LO (Lammers et al. 1996).

MAP kinase assay: Cell will be treated with growth factors and/or inhibitors, extracted with buffer and assayed for enzyme activity using a MAP kinase specific substrate peptide according to the method of Das and Vonderhaar (1996).

In addition the proteins will be resolved by SDS-PAGE, transferred to nitrocellulose and probed with a specific antibody to MAP kinase.

RESULTS AND DISCUSSION:

Signal Transduction of Bioactive Lipids in Breast cancer is being investigated in Hs 578T cell line.

We have shown in the tumor line IGF-1, a growth factor, activates both MAP kinase and

5-Lipoxygenase during signal transduction. We have assayed the enzyme activity by analysing the phosphorylation of these proteins on a Western Blot using specific antibodies against each kinase. The normal counter part of this breast cancer line Hs 578Bst was also examined. In Hs 578T breast cancer line we have shown that IGF-1 activates both the ras-MAPK and the 5-Lipoxygenase pathway for signal transduction.

a) TIME COURSE OF MAPK ACTIVATION BY IGF-1

Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphotyrosine antibody and analysed on a 10% SDS-gel. The blot was probed with Erk-2 antibody and detected with ECL kit. MAPK is also phosphorylated at the tyrosine residue of the enzyme where it reaches its peak within 5 mins of IGF-1 treatment.

b) TIME COURSE OF RAF-1 and MEK ACTIVATION BY IGF-1

Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphothreonine antibody and analysed on a 10% SDS-gel. The blot was probed with raf-1 or MEK antibody and detected with ECL kit. Raf-1 and MEK-1 is also rapidly activated, within 1-5 mins. of IGF-1 treatment.

c) TIME COURSE OF 5-LO ACTIVATION BY IGF-1

Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphotyrosine antibody and analysed on a 10% SDS-gel. The blot was probed with 5-LO antibody and detected with ECL kit. 5-LO, the enzyme involved in the Arachidonic acid pathway is very rapidly activated, reaching its peak within 1-5 mins of IGF-1 exposure.

d) EFFECT OF 5-LO INHIBITORS ON MAPK ACTIVATION BY IGF-1

Various inhibitors of these pathways are being investigated on the activation of these kinases. After serum starvation cells were preincubated with the inhibitors (MK 591, MK886, Curcumin,

NDGA) for 30 min prior to treatment with IGF-1. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel. The inhibitors of the 5-LO pathway were able to block the activation of MAPK by IGF-1.

e) EFFECT OF 5-LO INHIBITORS ON SHC ACTIVATION:

f) EFFECT OF RAS-MAPK INHIBITORS ON 5-LO ACTIVATION:

After serum starvation cells were preincubated with the inhibitors (FTase, PD98059 10μM. PD98059 50μM, LY294002) for 30 min prior to treatment with IGF-1. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel. The western blot was probed with 5-LO antibody.The inhibitors of the ras-MAPK pathway were not able to block the activation of 5-LO by IGF-1.

Thus the activation of 5-LO is upstream of activation of MAPK

f) EFFECT OF 5-LO INHIBITORS ON MAPK ACTIVATION BY INSULIN

Cells were also treated with Insulin and similar study was carried out with these breast cancer cells. After serum starvation cells were preincubated with the inhibitors of AA pathway (MK886, PCA, Curcumin) for 30 min prior to treatment with insulin. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel. Cells were able to activate MAPK in the presence of Insulin and this activation was blocked in when preincubated with 5-LO inhibitors.

g) RT-PCR of 5-LO IN THE PRESENCE OF INHIBITORS AND IGF-1:

These data suggest that in breast cancer cells, IGF which acts as a mitogen transduces its signal through multiple signaling pathways. This growth factor activates MAPK which is a well

known pathway for mitogenesis, however in the present study we show that both the AA pathway and the ras-MAPK pathway is activated in the presence of IGF-1. The inhibitors of the 5-LO pathway are able to completely block the IGF-1 induced stimulation of MAPK suggessting a possible cross talk between the two important signaling pathways. In the presence of MAPK inhibitors however the activation of 5-LO was not blocked indicating that 5-LO is upstream of MAPK signaling. We are analysing the AA metabolites currently in the presence of these various inhibitors which will give us a better understanding of the cross talk between the two pathways.

FIGURE LEGENDS:

FIG. 1. TIME COURSE OF MAPK ACTIVATION BY IGF-1
Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphotyrosine antibody and analysed on a 10% SDS-gel. The blot was probed with Erk-2 antibody and detected with ECL kit.

FIG. 2. TIME COURSE OF RAF-1 ACTIVATION BY IGF-1
Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphothreonine antibody and analysed on a 10% SDS-gel. The blot was probed with raf-1 antibody and detected with ECL kit.

FIG. 3. TIME COURSE OF MEK ACTIVATION BY IGF-1
Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphothreonine antibody and analysed on a 10% SDS-gel. The blot was probed with Mek-1 antibody and detected with ECL kit.

FIG. 4. TIME COURSE OF 5-LO ACTIVATION BY IGF-1
Cells were serum starved for 48hrs and treated with IGF-1 for different time periods, cell extract was immunoprecipitated with anti-phosphotyrosine antibody and analysed on a 10% SDS-gel. The blot was probed with 5-LO antibody and detected with ECL
kit.

FIG. 5. EFFECT OF 5-LO INHIBITORS ON MAPK ACTIVATION BY IGF-1
After serum starvation cells were preincubated with the inhibitors (MK 591, MK886) for 30 min prior to treatment with IGF-1. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel.

FIG.6. EFFECT OF RAS-MAPK INHIBITORS ON 5-LO ACTIVATION BY IGF

After serum starvation cells were preincubated with the inhibitors (FTase, PD98059 10μM. PD98059 50μM, LY294002) for 30 min prior to treatment with IGF. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel. The western blot was probed with 5-LO antibody.

FIG. 7. EFFECT OF 5-LO INHIBITORS ON MAPK ACTIVATION BY INSULIN
After serum starvation cells were preincubated with the inhibitors (MK886, PCA, Curcumin) for 30 min prior to treatment with insulin. Cell were homogenised and the extract immunoprecipitated and analysed on a 10% SDS gel.

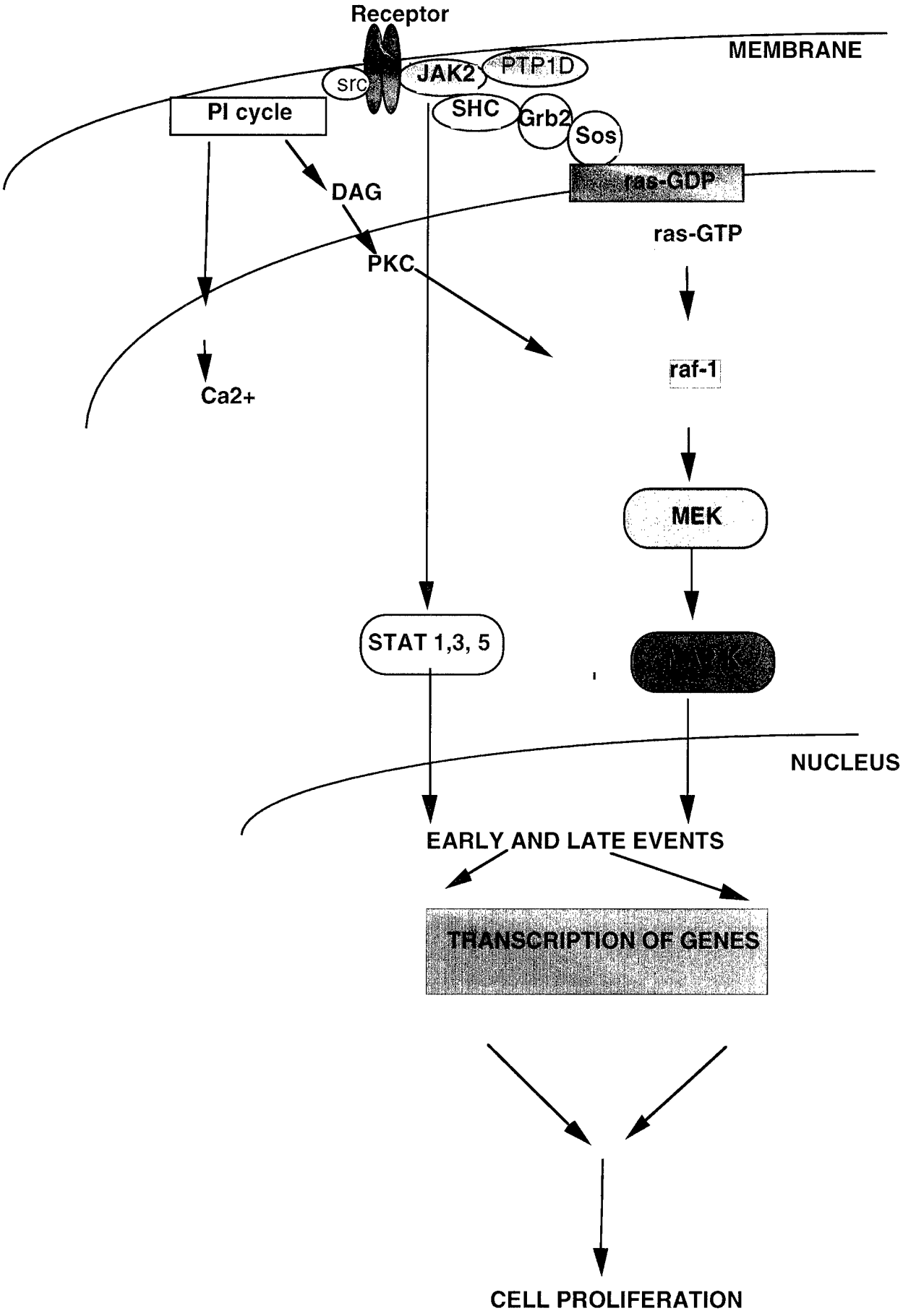

Receptor
MEMBRANE
src
JAK2
PTP1D
SHC
Grb2
Sos
PI cycle
DAG
ras-GDP
ras-GTP
PKC
Ca2+
raf-1
MEK
STAT 1,3, 5
NUCLEUS
EARLY AND LATE EVENTS
TRANSCRIPTION OF GENES
CELL PROLIFERATION

FIGURE 1

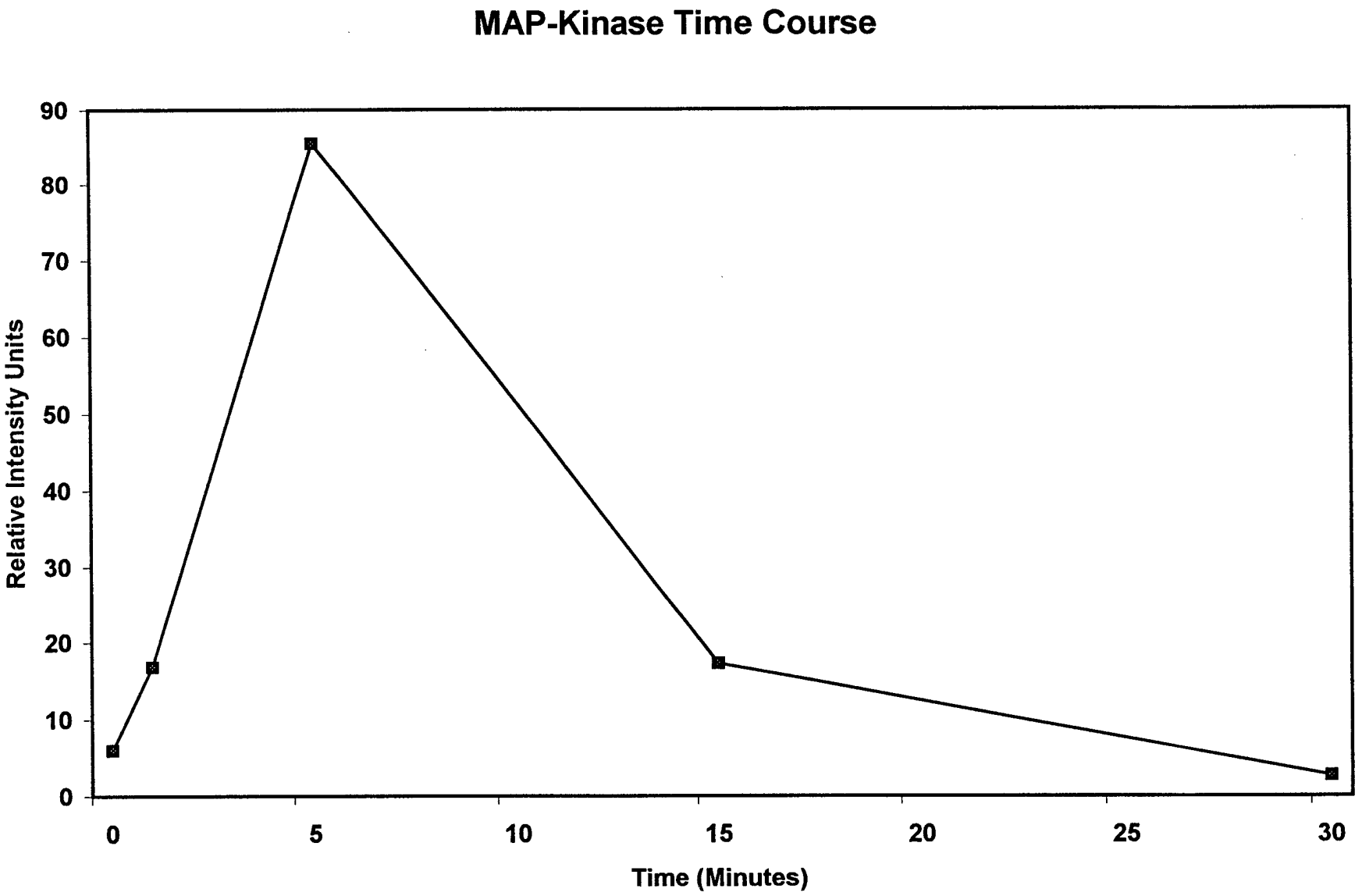
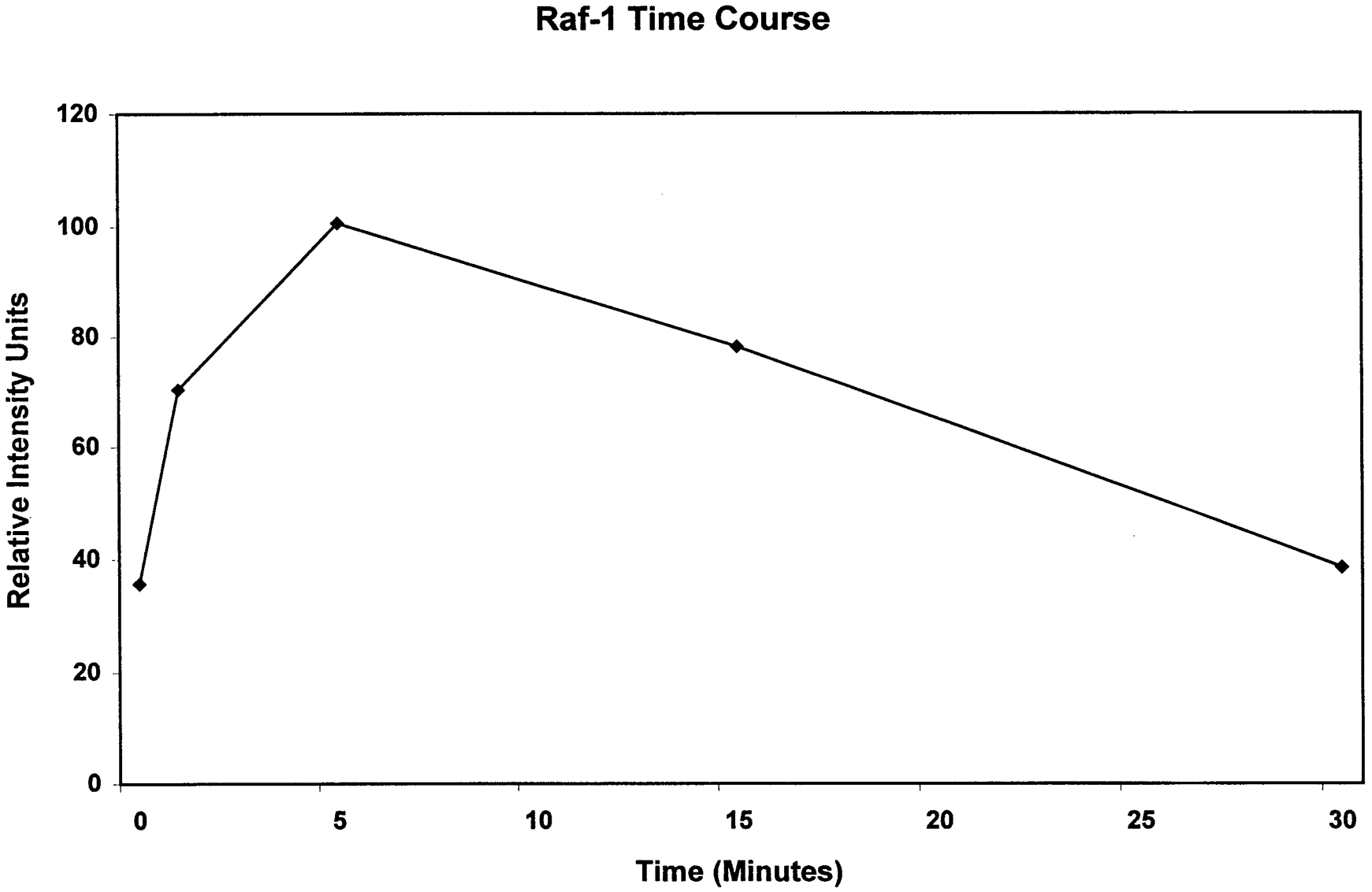

MAP-Kinase Time Course
Relative Intensity Units
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (Minutes)

FIGURE 2


Raf-1 Time Course
Relative Intensity Units
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (Minutes)

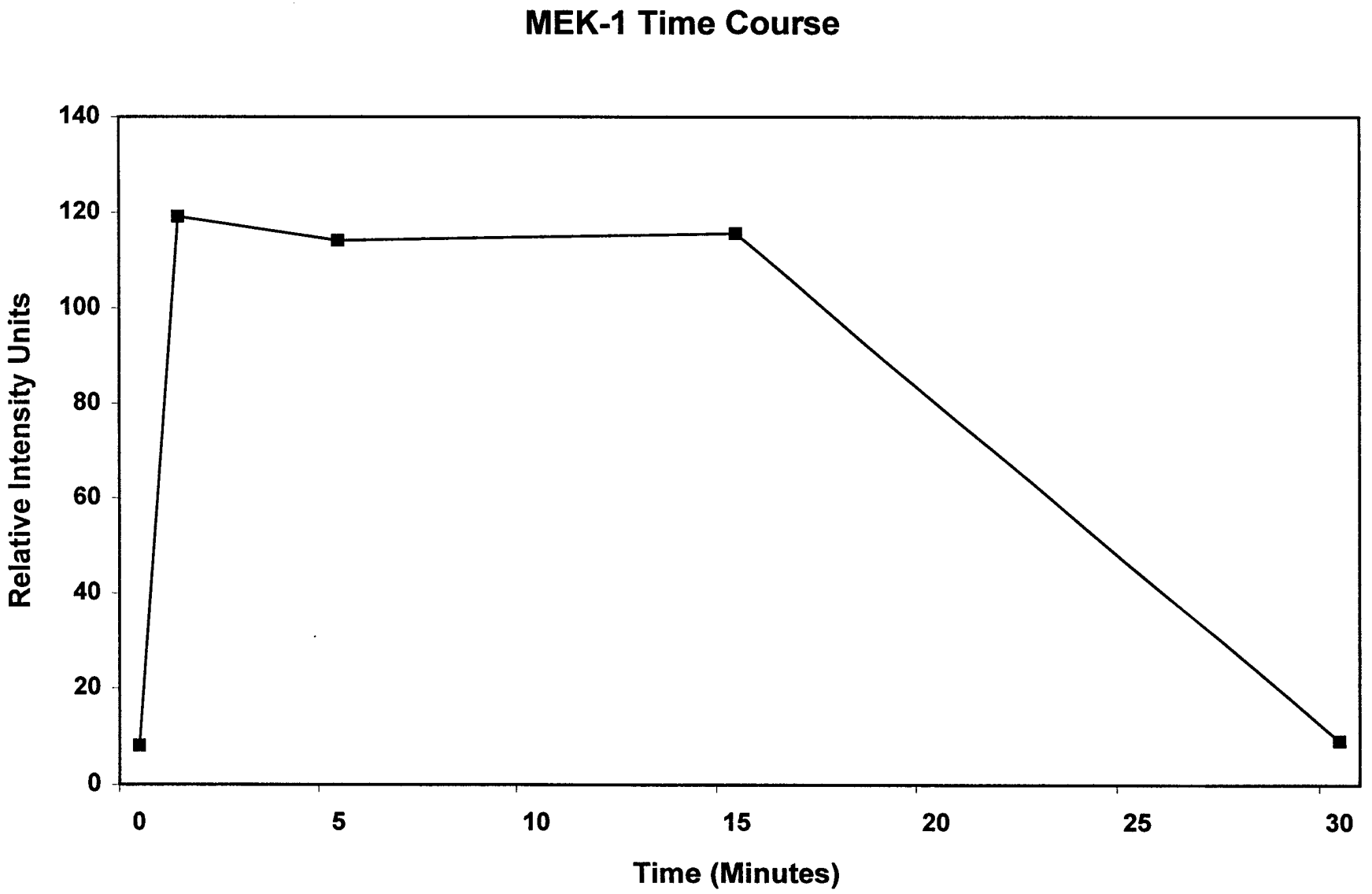

MEK-1 Time Course
Relative Intensity Units
140
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (Minutes)

FIGURE 4

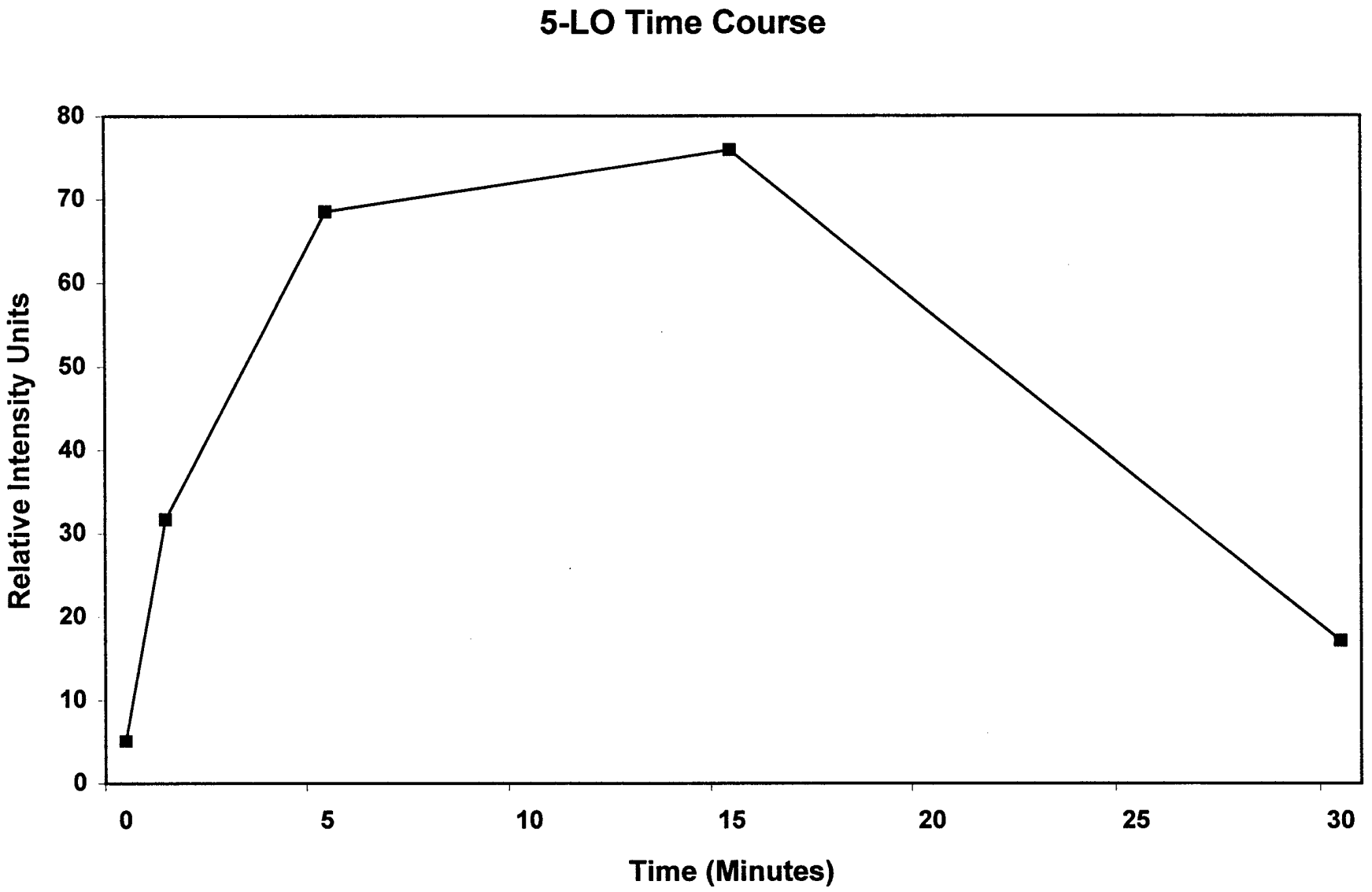
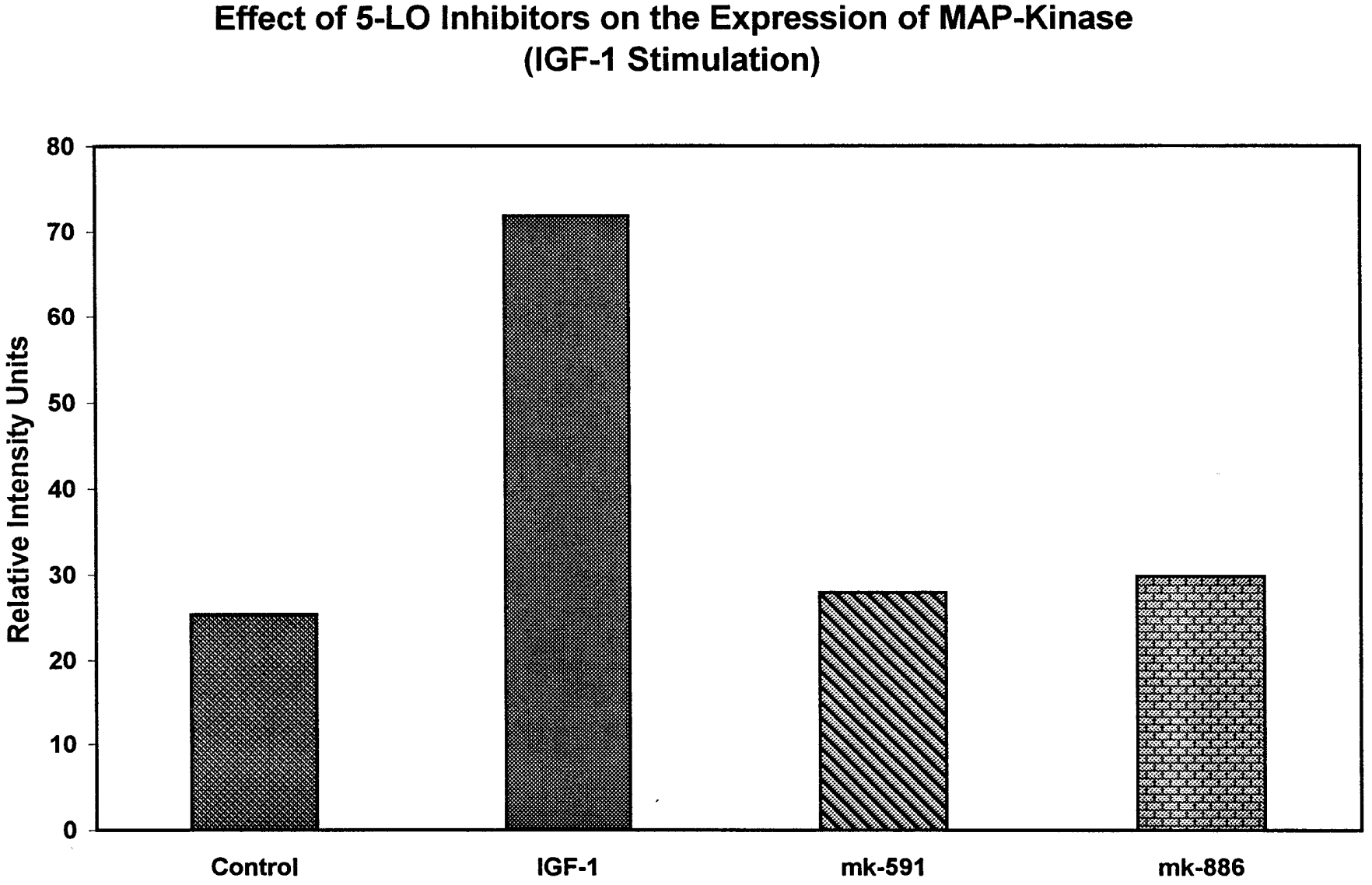

5-LO Time Course
Relative Intensity Units
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
Time (Minutes)

FIGURE 5


Effect of 5-LO Inhibitors on the Expression of MAP-Kinase
(IGF-1 Stimulation)
Relative Intensity Units
80
70
60
50
40
30
20
10
0
Control
IGF-1
mk-591
mk-886

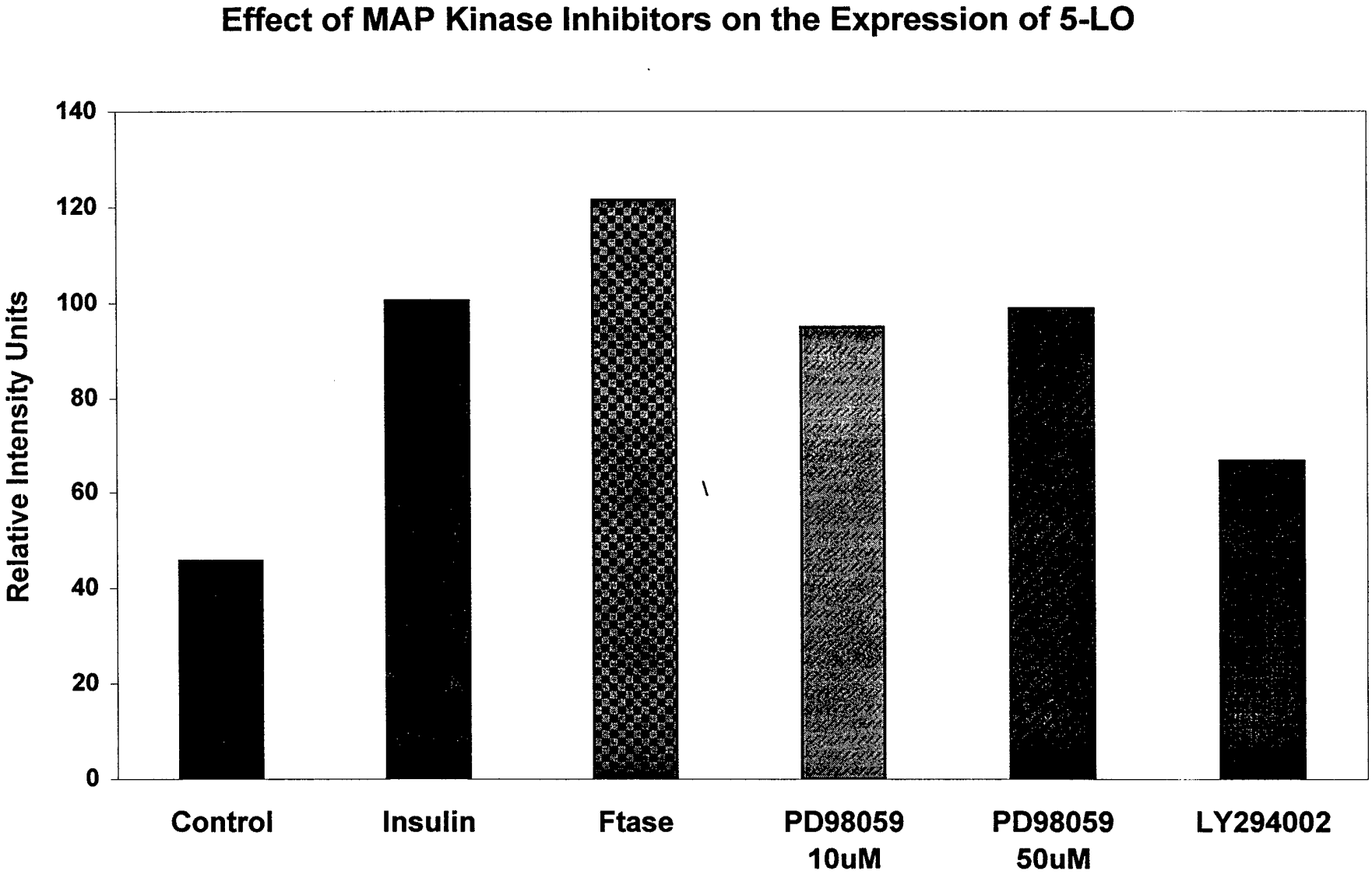

Effect of MAP Kinase Inhibitors on the Expression of 5-LO
Relative Intensity Units
140
120
100
80
60
40
20
0
Control
Insulin
Ftase
PD98059 10uM
PD98059 50uM
LY294002

FIGURE 6B

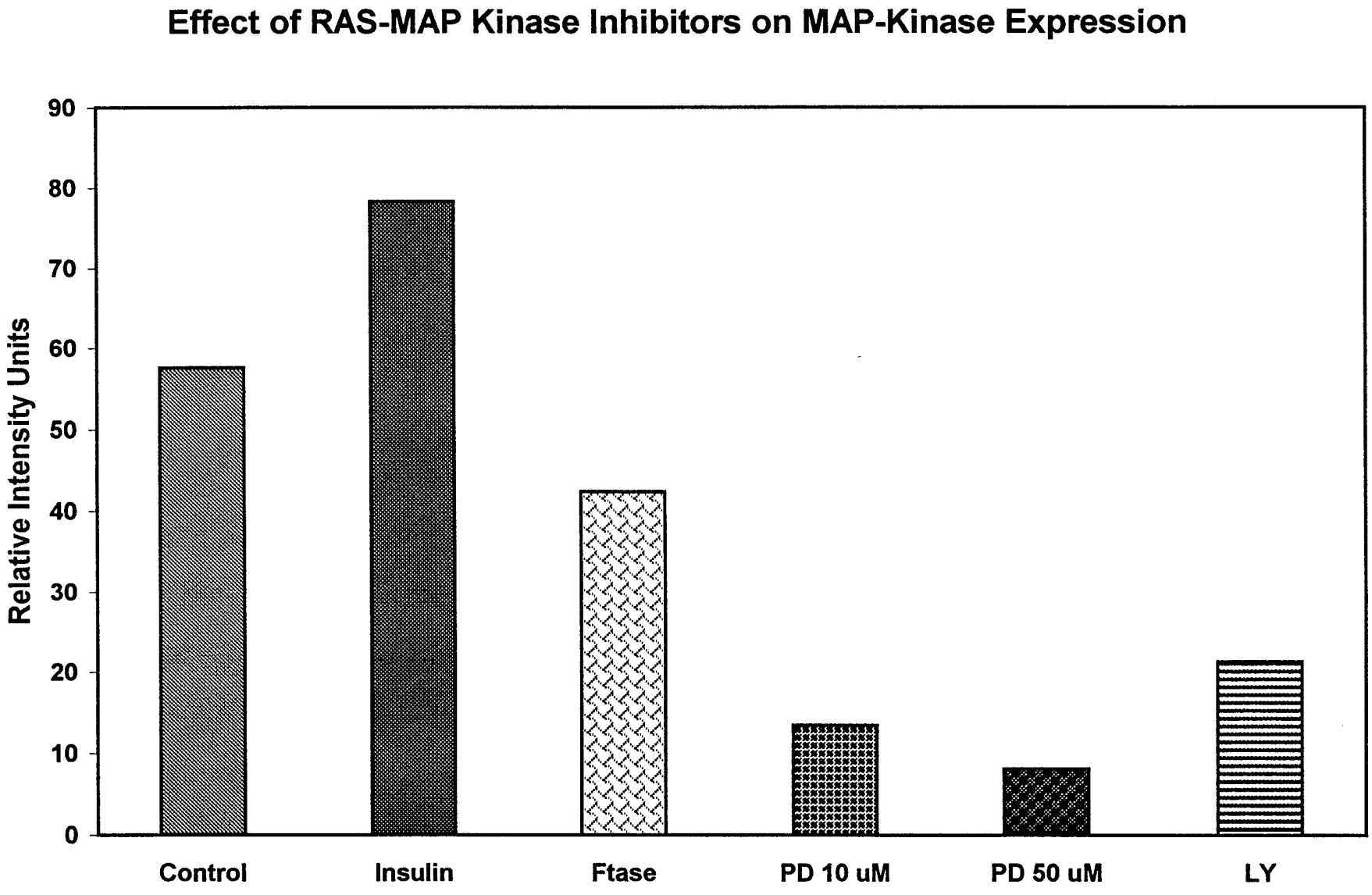
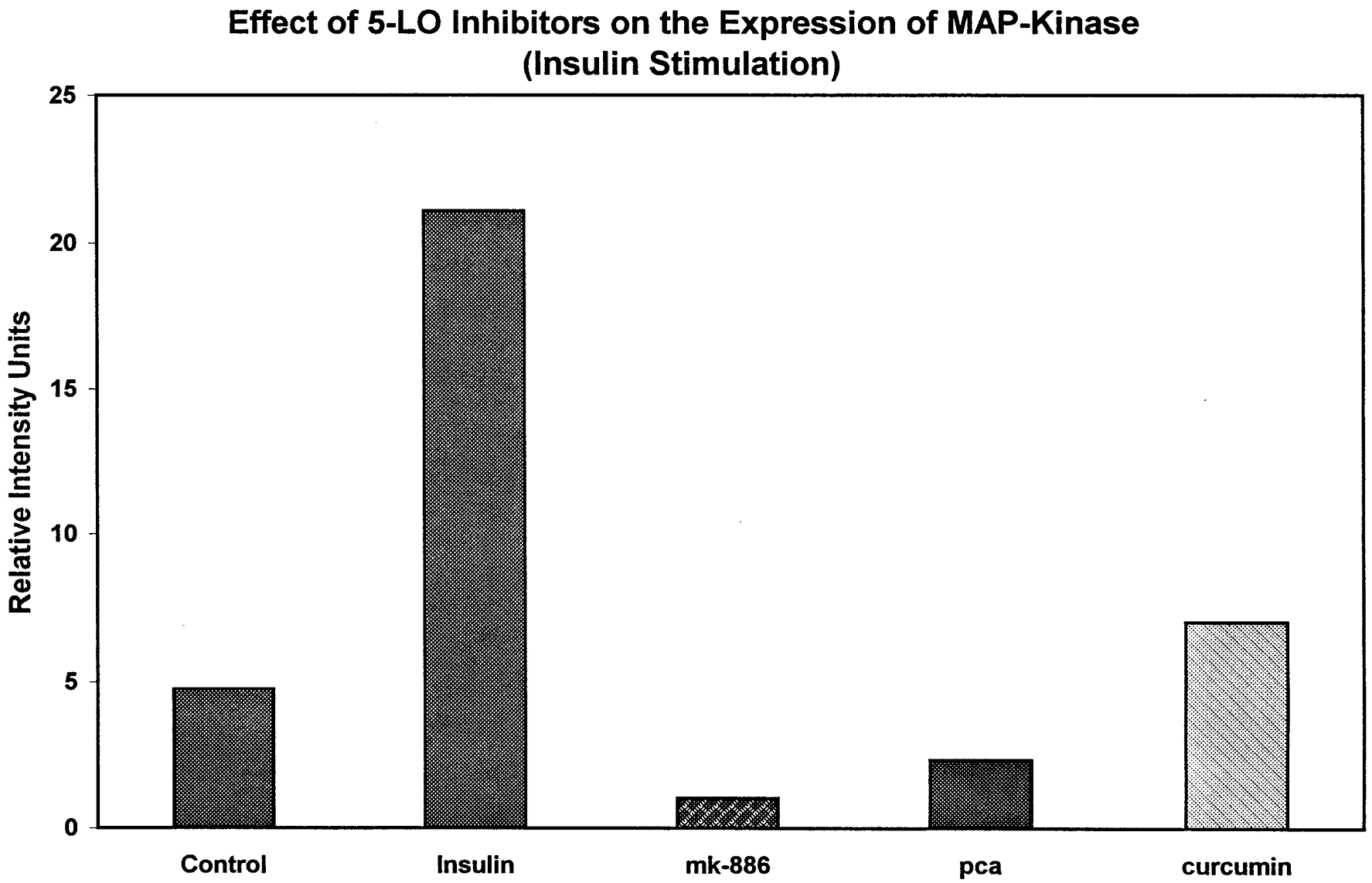

Effect of RAS-MAP Kinase Inhibitors on MAP-Kinase Expression
Relative Intensity Units
90
80
70
60
50
40
30
20
10
0
Control
Insulin
Ftase
PD 10 uM
PD 50 uM
LY

FIGURE 7


Effect of 5-LO Inhibitors on the Expression of MAP-Kinase
(Insulin Stimulation)
Relative Intensity Units
25
20
15
10
5
0
Control
Insulin
mk-886
pca
curcumin